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Aberrant methylation of imprinted genes is associated with negative hormone receptor status in invasive breast cancer

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Epigenetic regulation of imprinted genes enables monoallelic expression according to parental origin, and its disruption is implicated in many cancers and developmental disorders. The expression of hormone receptors is significant in breast cancer because they are indicators of cancer cell growth rate and determine response to endocrine therapies. We investigated the frequency of aberrant events and variation in DNA methylation at nine imprinted sites in invasive breast cancer and examined the association with estrogen and progesterone receptor status. Breast tissue and blood from patients with invasive breast cancer ($n = 38$) and benign breast disease ($n = 30$) were compared with those from healthy individuals ($n = 36$), matched with the cancer patients by age at diagnosis, ethnicity, body mass index, menopausal status and familial history of cancer. DNA methylation and allele-specific expression were analyzed by pyrosequencing. Tumor-specific methylation changes at *IGF2 DMR2* were observed in 59% of cancer patients, *IGF2 DMR0* in 38%, *DIRAS3 DMR* in 36%, *GRB10 ICR* in 23%, *PEG3 DMR* in 21%, *MEST ICR* in 19%, *H19 ICR* in 18%, *KvDMR* in 8% and *SNRPN/SNURF ICR* in 4%. Variation in methylation was significantly greater in breast tissue from cancer patients compared with that in healthy individuals and benign breast disease. Aberrant methylation of three or more sites was significantly associated with negative estrogen-alpha (Fisher's exact test, $p = 0.02$) and progesterone-A ($p = 0.02$) receptor status. Aberrant events and increased variation in imprinted gene DNA methylation, therefore, seem to be frequent in invasive breast cancer and are associated with negative estrogen and progesterone receptor status, without loss of monoallelic expression.

Cancer Genetics

Key words: DNA methylation, genomic imprinting, pyrosequencing, breast cancer, hormone receptor

Abbreviations: CBCP: Clinical Breast Care Project; CpG: cytosine–phosphate–guanine; DMR: differentially methylated region; ER: estrogen receptor; FAM: frequently altered methylation; HER2: human epidermal growth factor receptor 2; ICR: imprinting control region; LOI: loss of imprinting; PR: progesterone receptor

Additional Supporting Information may be found in the online version of this article.

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Genomic imprinting is the epigenetic regulation of genes to enable monoallelic expression according to parental origin, through differential methylation of regions labeled imprinting control regions (ICRs) when established in the germline, or differentially methylated regions (DMRs) when established postfertilization. Loss of imprinting (LOI) is the loss of this monoallelic expression, and it is commonly the result of disruption of DNA methylation at ICRs and DMRs. LOI is associated with a range of disorders, such as the Beckwith–Wiedemann, Angelman and Prader–Willi syndromes.[1,2] Imprinted genes have also been implicated in a range of cancers, including those of the breast[3] and ovaries.[4] Their expression has been associated with disease progression, including reduced survival in pancreatic cancer[5] and aggressive prostate cancers.[6] Aberrant methylation of imprinted genes may be an early event involved in neoplastic transformation,[7] and it has been reported that 10% of apparently healthy individuals display LOI of *IGF2*.[8]

DNA methylation can display stochastic variation, which may facilitate developmental plasticity and adaptation to environments, including that of malignant cells in the tumor microenvironment.[9] This variation can be gene-specific, such

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What's new?

Epigenetic regulation of imprinted genes enables monoallelic expression according to parental origin, and its disruption is implicated in many cancers. Elucidating the relationship between imprinted genes and hormone receptor status in breast cancer may provide insight into tumorigenesis and potential prognostic factors. This is the first study to identify an association between the aberrant DNA methylation of imprinted genes and negative status of the estrogen and progesterone receptors in breast cancer. Variation in methylation increases from normal tissue to benign disease to cancer. Epigenetic disruption of imprinted genes may play an important role in the development of different breast cancer subtypes.

as that observed in the placenta to enable adaptation to environmental challenges throughout pregnancy.[10] A significantly greater level of variation is observed in tumors, which often constitutes shifting in methylation "boundaries" between cytosine–phosphate–guanine (CpG) islands and shores.[11] Such events may occur early in tumorigenesis, demonstrated by the significantly increased variation observed in cervical cells of normal morphology.[12]

Breast cancers are classified according to the expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). ER-alpha and PR-A expressions are determined by immunohistochemistry, with tumors in which [mt]5–10% of cells stain positively are classified as positive for expression. HER2 expression is determined by immunohistochemistry, with scores of 0–3 according to intensity of staining, and by fluorescence *in situ* hybridization to detect amplification of the gene. Activation of these receptor signaling pathways results in cellular proliferation, and they are implicated in the progression of breast and gynecologic cancers. Approximately two-thirds of tumors display expression of at least one of the hormone receptors, and these patients display reduced mortality compared with those who express neither,[13] in part due to the efficacy of endocrine therapies. Triple-negative breast cancers, which account for approximately 12–17% of all cases,[14] are associated with poor prognosis.

The relation between imprinted genes and hormone receptor status in breast cancer has not been well elucidated. Correlations between the methylation of four tumor-suppressor genes and the expression of the hormone receptors have been observed,[3] but similar studies have not been performed for imprinted genes. Elucidating such a relationship may provide insight into tumorigenesis and the determination of prognostic factors.

In this study, we investigated the aberrant methylation of nine imprinted regions in samples of breast tissue taken from healthy individuals and patients with benign breast disease and invasive breast cancer. The interrogated imprinted regions were: *DIRAS3 DMR*; *GRB10 ICR*; *H19 ICR*; *IGF2 DMR0* and *DMR2*; *KvDMR; MEST ICR*; *PEG3 DMR* and *SNPRN/SNURF ICR*. The intraindividual tissue-specificity of the aberrant methylation events was determined by comparison of methylation in DNA from breast tissue and peripheral blood within individuals. Variation in DNA methylation in imprinted genes and LINE-1 global methylation were measured, and possible associations between aberrant methylation and the status of the estrogen, progesterone and HER2 receptors in breast cancer patients were identified. Finally, we analyzed the allele-specific expression of the genes to establish the relative impact of the aberrant methylation events.

Material and Methods

Study populations

The Clinical Breast Care Project (CBCP) is a clinical and research program that began enrolling patients in 2001. The primary clinical arm of the CBCP was the Clinical Breast Care Center at Walter Reed Army Medical Center (Washington DC). Additional recruitment centers include the Joyce Murtha Breast Care Center (Windber, PA) and the Anne Arundel Medical Center (Annapolis, MD). Enrolled patients were required to be 18 years old or older, mentally competent and willing to provide informed consent, and presenting with evidence of possible breast disease, attending for routine screening mammograms, or undergoing elective reductive mammoplasty. Patients were provided with layered consent forms that included permission to obtain samples of blood, breast and metastatic tissues, and a description of the primary research uses. Once informed consent was granted, the core questionnaire, with over 500 fields of information, was completed with the help of a nurse case manager, and an extensive pathology checklist was completed by the dedicated breast pathologist. Ethical approval for the collection of blood and tissue samples and their use in this study was provided by the Walter Reed Army Medical Center Human Use Committee and Institutional Review Board.

The Susan G. Komen for the Cure Tissue Bank (Indianapolis, IN) is a charitable organization that enrolls healthy volunteers to donate blood and up to four breast biopsies. Eligible individuals must be at least 18 years of age and able to provide informed consent. Volunteers are asked to complete a questionnaire regarding health and lifestyle factors. Approval for the collection of blood and tissue, and for their use in this study, was provided by the Indiana University Institutional Review Board.

Blood and tissue samples

DNA and RNA from benign and tumor tissue and blood were obtained from the CBCP for 38 patients with invasive breast cancer and 30 with benign breast disease (13 fibrocystic changes, 8 fibroadenoma, 3 postsurgical changes, 2

Table 1. Characteristics of the participants

	Healthy	Benign breast disease	Invasive cancer
Number of participants	36	30	38
Age at diagnosis, years	50.3	47.5	51.5
Ethnicity, *n* (%)			
White	29 (80.6)	21 (70.0)	28 (73.7)
African-American	7 (19.4)	5 (16.7)	7 (18.4)
Other	0 (0.0)	4 (13.3)	3 (7.9)
BMI	28.0	25.2	28.5
Menopausal status, *n* (%)			
Premenopausal	21 (58.3)	20 (66.7)	19 (50.0)
Postmenopausal	15 (41.7)	10 (33.3)	19 (50.0)
Familial history of cancer, *n* (%)	17 (48.6)	11 (36.7)	18 (47.4)

The characteristics of the individuals involved in the study from whom samples of breast tissue and blood were taken. Healthy individuals were matched to the cancer patients by age, ethnicity, BMI, menopausal status and familial history of cancer. The ages and BMIs are the means of each category. Postmenopausal status includes patients who were surgically postmenopausal, such as following hysterectomy. Familial history of cancer refers to primary and secondary history. For one healthy individual, the familial history was not known. BMI: body mass index.

stromal fibrosis and 4 others) (Table 1 and Supporting Information Table 1). Genomic DNA was extracted from frozen tumor samples following laser microdissection (Leica Microsystems, Wetzlar, Germany) and from homogenized benign tissue by means of incubation of the samples with proteinase K at 37 °C overnight before passage through purification columns (Millipore, Billerica, MA). DNA extractions from blood were performed using Clotspin and Puregene DNA purification kits (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Samples from 36 healthy individuals were obtained from the Susan G. Komen for the Cure Foundation Tissue Bank (Table 1). DNA was extracted from 25 mg tissue using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol, following proteinase K digestion at 56 °C for 3–6 hrs. RNA extractions were performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol, following mechanical disruption of 25 mg tissue in a bead mill. Between 2 and 41 µg of DNA isolated from blood was made available for this study.

Bisulfite conversion of DNA

About 250–500 ng of DNA was converted using the EZ DNA Methylation Gold kit (Zymo Research, Irvine, CA), according to the manufacturer's Alternative 2 protocol, with elution in 40 µl of elution buffer.

DNA methylation analysis by pyrosequencing

Pyrosequencing was performed on the Pyromark Q24 Pyrosequencer (Qiagen). Assays were designed using the PyroMark Assay Design and PyroMark Q24 software programs (Qiagen), with the exceptions of those for *H19 ICR*, *IGF2 DMR0* and *IGF2 DMR2*, which have previously been reported.[15–17] Nine DMRs/ICRs were analyzed: *DIRAS3 DMR* (five CpG dinucleotides); *GRB10 ICR* (six CpGs); *H19 ICR* (eight CpGs); *IGF2 DMR0* (six CpGs); *IGF2 DMR2* (seven CpGs); *KvDMR* (nine CpGs); *MEST ICR* (nine CpGs); *PEG3 DMR* (six CpGs) and *SNRPN/SNURF ICR* (eight CpGs) (Supporting Information Table 2).

Regions of interest were amplified by polymerase chain reaction, using 3 µl of bisulfite-treated DNA and 0.2 µM of each primer with HotStar Taq Plus Master Mix (Qiagen) in a final volume of 20 µl. Pyrosequencing was performed according to the manufacturer's instructions. Duplicate bisulfite-conversions were run for each sample, and mean methylation levels were calculated across all CpG sites per replicate. Studies in healthy human tissues have reported methylation levels of between 30% and 70% at DMRs and ICRs,[16,17] and we, therefore, defined hypomethylation as values below 30% and hypermethylation as values above 70%. It was not possible to ascertain methylation values for all samples.

Identification of allelic origins of mRNA

Allele-specific expression was performed by pyrosequencing, using single-nucleotide polymorphisms (SNPs) to determine the allelic origins of mRNA transcripts in heterozygous patients, identified by pyrosequencing using 10 ng of DNA from blood. For heterozygous individuals, 20 ng of RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions, and 2 µl of cDNA used for polymerase chain reaction-based amplification prior to allele quantification by pyrosequencing. Primer sequences and SNPs are provided in Supporting Information Table 2.

Gene expression microarray data

Expression data for ten genes (*DIRAS3*, *DNMT1*, *DNMT3A*, *DNMT3B*, *GRB10*, *IGF2*, *KCNQ1*, *MEST*, *PEG3* and *SNRPN*) from 302 breast tumors were made available by the Walter Reed Army Medical Center (Washington DC). Of the tumors, 199 were ER-positive and 103 were ER-negative, and 153 were PR-positive and 149 PR-negative.

Statistical analysis

Correlations between DNA methylation and age at diagnosis were calculated by Pearson's correlation for all genes except *GRB10*, for which Spearman's rank correlation was used because the data were not normally distributed. Associations with tumor stage, receptor status and familial history of cancer were calculated by analysis of variance. For the frequently altered methylation (FAM) group, associations were calculated by Fisher's exact test, and associations with age at diagnosis were calculated by t-test or Wilcoxon rank-sum test, according to the distribution of the data. The p-values below 0.05 were deemed statistically significant.

For the modeling of variation, deviation was calculated as the absolute difference between the median methylation level in blood or breast tissue from healthy individuals and the methylation level for the individual. This was conducted for each gene, for comparisons of samples from patients with benign breast disease and cancer with the healthy controls, in both blood and breast tissue. A general linearized model was used to model deviation with a gamma distribution, and a log link. Model coefficients and 95% confidence intervals were exponentiated to give the relative change in deviation between tissues. The adjusted model controlled for family history (binary), menopausal status (binary), body mass index (BMI) category (normal, <25; overweight, 25–30 and obese, >30) and age (continuous). Results were corrected for multiple hypothesis testing using the Bonferroni correction, and *p*-values below 0.05 were deemed statistically significant.

Results

Aberrant DNA methylation of imprinted genes is a frequent event in breast tumors

We investigated the DNA methylation at nine imprinted regions in breast tissue and peripheral blood from 36 healthy individuals, 30 patients with benign breast disease and 38 with invasive cancer. We have previously reported methylation levels at six of the imprinted regions (*GRB10 ICR*, *H19 ICR*, *IGF2 DMR0*, *IGF2 DMR2*, *KvDMR* and *SNRPN/SNURF ICR*) in patients with benign breast disease and breast cancer,[18] and we integrated these results with methylation levels measured in healthy individuals to identify the relative frequency of aberrant methylation (hypo- and hypermethylation) in the disease states.

In breast tissue, median methylation levels were close to the expected 50%, with the exception of the *IGF2* sites, which also displayed greater disparities in the median levels between normal, benign and tumor tissue (Fig. 1). Aberrant methylation was frequently observed in invasive breast cancer, observed at *IGF2 DMR2* in 59% of patients, *IGF2 DMR0* in 38%, *DIRAS3 DMR* in 36%, *GRB10 ICR* in 23%, *PEG3 DMR* in 21%, *MEST ICR* in 19%, *H19 ICR* in 18%, *KvDMR* in 8% and *SNRPN/SNURF ICR* in 4% (Supporting Information Table 3). Hypomethylation was more common than hypermethylation at all sites except *MEST ICR* and *PEG3 DMR*, and was not associated with whether the genes are maternally or paternally expressed. Among patients with benign breast disease, aberrant methylation was only observed at *IGF2 DMR2*, which was hypomethylated in 12 of the 26 successfully analyzed samples. No aberrant methylation was observed in healthy individuals at seven of the imprinted regions, with *IGF2 DMR0* hypermethylated in one sample and *IGF2 DMR2* hypomethylated in three.

To determine the intraindividual tissue specificity of the methylation changes observed in breast tumors, we analyzed peripheral blood samples taken from the same individuals. Median values for all nine regions were between 39.1% and 53.6% across the three groups (Supporting Information Table 3). No samples met the criteria of being hypo- or hypermethylated, indicating that the aberrant methylation events were unique to breast tissue.

Variation in DNA methylation is significantly greater in breast tissue and blood from patients with invasive breast cancer and benign breast disease

Variation in DNA methylation in the healthy and disease states was estimated by the absolute difference between the methylation level in an individual and the median level measured in breast tissue or peripheral blood from healthy individuals, with the latter representing the normal value. The mean deviation from the median methylation levels observed in normal breast tissue was greatest in invasive tissue, ranging from 6.6% for *SNRPN/SNURF ICR* to 23.0% for *IGF2 DMR0* (Fig. 2a and Supporting Information Table 4A). The mean deviations were lowest in normal breast tissue, where they were under 2.0%, with the exceptions of *IGF2 DMR0* (4.2%) and *IGF2 DMR2* (5.5%). Benign breast tissue displayed intermediate values, with median deviation ranging from 2.5% for *DIRAS3 DMR* to 11.2% for *IGF2 DMR2.*

The mean deviations in healthy individuals were lower in blood than in breast tissue, with the exceptions of *PEG3 DMR* in healthy individuals and *DIRAS3 DMR* in patients with benign breast disease (Fig. 2a and Supporting Information Table 4A). The deviations were greatest in blood from invasive breast cancer patients, where median deviations ranged between 1.7% and 6.6%. The values ranged between 1.5% and 6.7% among benign breast disease patients, and between 0.7% and 3.2% in healthy individuals.

The relative deviations from the median methylation levels were significantly greater at all interrogated regions in tumor tissue than in normal breast tissue, ranging from 2.6-fold (*IGF2 DMR2*) to 9.7-fold (*DIRAS3 DMR*) greater (Fig. 2b and Supporting Information Table 4B). When the model was adjusted for the age, BMI, menopausal status and familial history of cancer of the individuals, statistical significance was retained at all sites. Variation was also significantly greater in benign breast disease tissue than in normal breast tissue for all DMRs except *DIRAS3 DMR*, with the relative deviations between 1.5-fold (*DIRAS3 DMR*) and 7.5-fold (*SNRPN/SNURF ICR*) greater. Significance was retained for all sites except *IGF2 DMR0* and *MEST ICR* with the adjusted model.

Significantly greater variation in methylation was similarly observed in peripheral blood. Mean deviations in blood from patients with invasive breast cancer were between 1.3-fold (*DIRAS3 DMR*) and 7.4-fold (*SNRPN/SNURF ICR*) greater than in healthy individuals and were significant for *GRB10 ICR*, *KvDMR*, *PEG3 DMR* and *SNPRN/SNURF ICR*. In the adjusted model, the relative changes were significant for *GRB10 ICR*, *IGF2 DMR2*, *PEG3 DMR* and *SNRPN/SNURF ICR* (Supporting Information Table 4B). Mean deviations were also between 1.3-fold (*IGF2 DMR0*) and 7.5-fold (*SNRPN/SNURF ICR*) greater in blood taken from patients

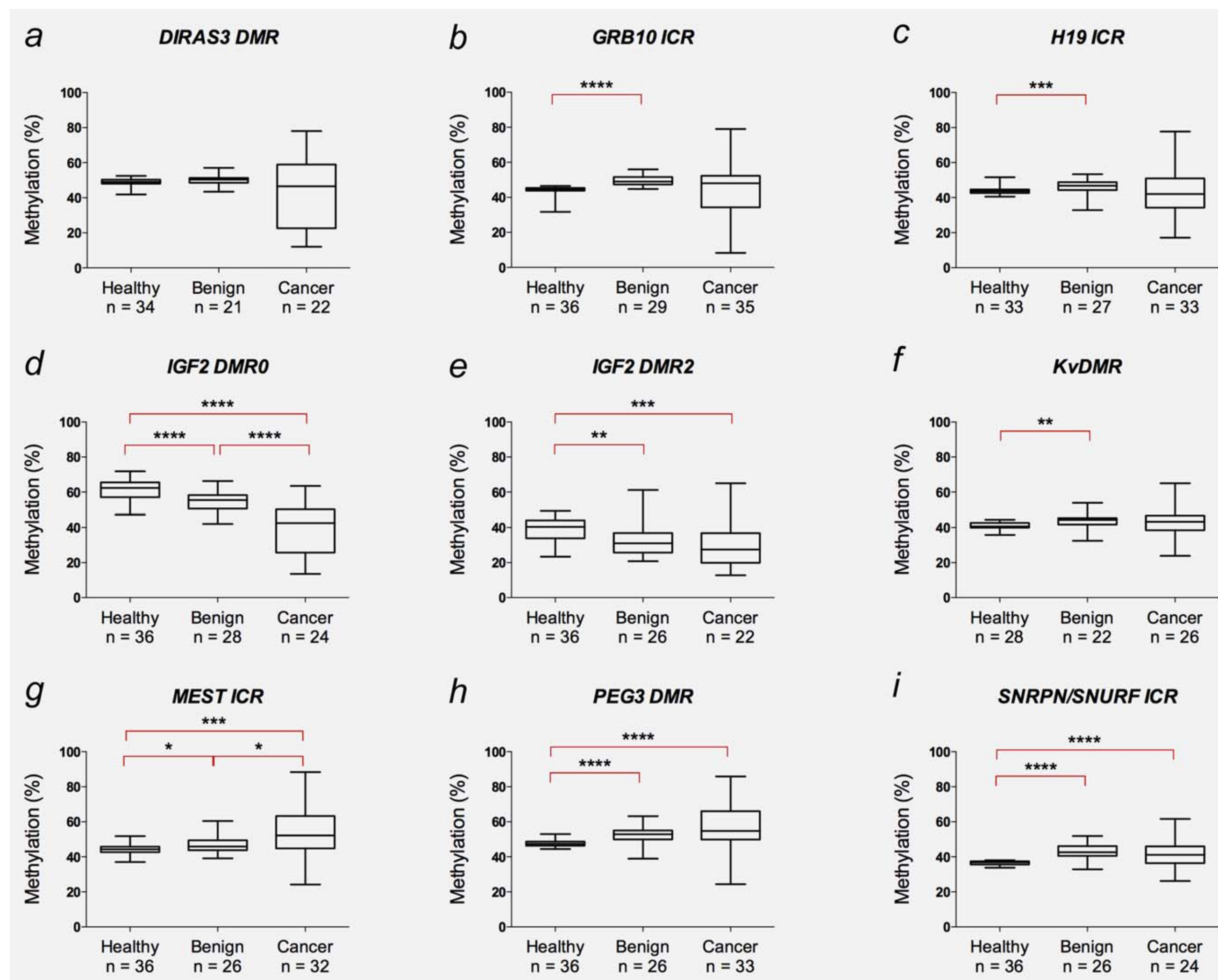

Figure 1. DNA Methylation at nine imprinted regions in breast tissue from patients and healthy individuals. Measured methylation values in breast tissue for *DIRAS3 DMR* (*a*), *GRB10 ICR* (*b*), *H19 ICR* (*c*), *IGF2 DMR0* (*d*), *IGF2 DMR2* (*e*), *KvDMR* (*f*), *MEST ICR* (*g*), *PEG3 DMR* (*h*) and *SNRPN/SNURF ICR* (*i*) in healthy individuals and patients with benign breast disease and invasive cancer. Boxes correspond to the median and interquartile range, and the whiskers to the full range of measured values. Statistically significant differences are indicated (Mann–Whitney *U*-test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.0005$ and ****$p < 0.0001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with benign breast disease than in healthy individuals (Fig. 2b and Supporting Information Table 4B). The differences were significant for *GRB10 ICR*, *MEST ICR*, *PEG3 DMR* and *SNRPN/SNURF ICR* in both the adjusted and unadjusted models (Supporting Information Table 4B).

Aberrant DNA methylation is associated with hormone receptor status

We investigated possible associations between the measured methylation values at each imprinted loci and expression of ER, PR and HER2 (details in Supporting Information Table 1). Hypermethylation of *PEG3 DMR* (analysis of variance, $p < 0.01$) and *IGF2 DMR0* ($p = 0.04$) were associated with negative ER status, whereas hypermethylation of *PEG3 DMR* ($p = 0.02$) and *MEST ICR* ($p = 0.03$) were associated with negative PR status (Table 2). No associations were observed between the methylation of imprinted genes and the expression of HER2 or tumor stage.

Frequently altered methylation and hormone receptor status

Twelve of the invasive tumors analyzed displayed aberrant methylation of at least three of the imprinted regions that were interrogated. We categorized these together as "frequently altered methylation" (FAM). FAM was associated with negative ER (Fisher's exact test, $p = 0.02$) and PR status ($p = 0.02$), but not HER2 ($p = 0.32$) or tumor stage ($p = 0.15$) (Table 2). All five triple-negative tumors displayed aberrant methylation at three or more sites.

Methylation at LINE-1 repetitive elements was measured in breast tissue from patients with benign breast disease and invasive cancer and analyzed according to the FAM grouping

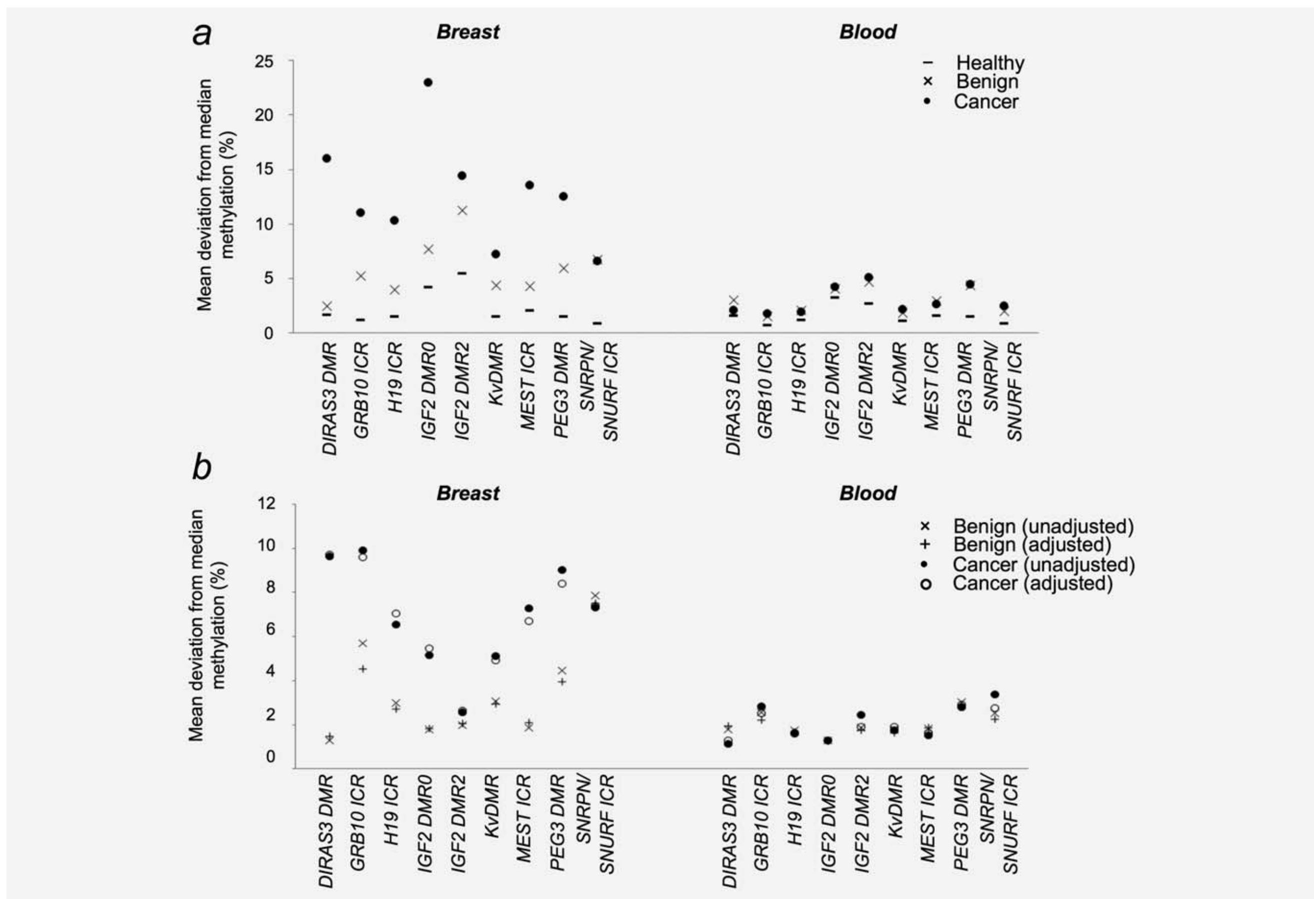

Figure 2. Variation in DNA methylation in breast tissue and blood. (*a*) Mean deviation from the measured median value in normal breast tissue or blood from healthy individuals for each of the nine imprinted regions. Results are from patients with invasive cancer (•), benign breast disease (×) and healthy individuals (−). (*b*) Change in mean deviation from median methylation values measured in normal tissue and blood, relative to values observed in normal breast tissue and blood from healthy individuals. Results for cancer patients from the unadjusted (•) and adjusted (○) models, and from patients with benign breast disease in the unadjusted (×) and adjusted (+) models.

Table 2. Correlations between DNA methylation of imprinted genes and tumor pathology

Imprinting regions	Age at diagnosis (ρ-values)	Tumor stage	ER	PR	ER/PR	HER2	Familial history
DIRAS3 DMR	0.88 (−0.04)	0.85	0.20	0.36	0.44	0.53	0.55
GRB10 ICR	0.96 (<0.01)	0.06	0.09	0.45	0.21	0.16	0.20
H19 ICR	0.39 (−0.16)	0.29	0.67	0.52	0.45	0.57	0.89
IGF2 DMR0	0.18 (−0.28)	0.15	**0.04**↓	0.98	**0.02**↓	0.22	0.23
IGF2 DMR2	0.49 (−0.15)	0.08	0.96	0.65	0.82	0.70	0.32
KvDMR	0.45 (−0.15)	0.24	0.70	0.31	0.59	0.33	0.38
MEST ICR	0.94 (−0.01)	0.58	0.23	**0.03**↓	0.10	0.40	0.42
PEG3 DMR	0.33 (−0.18)	0.71	**<0.01**↓	**0.02**↓	**0.01**↓	0.99	0.66
SNRPN ICR	0.58 (0.12)	0.89	0.89	0.46	0.56	0.61	0.66
FAM	0.91	0.15	**0.02** (−)	**0.02** (−)	**0.02** (−)	0.32	0.28

Correlations between aberrant methylation of nine imprinted genes and the tumor and patient characteristics were identified using Pearson's correlation (age at diagnosis) and analysis of variance (tumor stage, receptor status and familial history of cancer). For the FAM group (tumors with more than three aberrantly methylated imprinted regions), Fisher's exact test was used to identify associations with tumor stage, receptor status and familial history, whereas a *t*-test or Wilcoxon rank-sum test was used to identify associations with age at diagnosis. The *p*-values are provided with ρ-values below where appropriate. Statistically significant ($p < 0.05$) associations are highlighted in bold. Down arrows (↓) indicate reduced levels of methylation with expression of the estrogen or progesterone receptor. For the FAM grouping, minus symbols (−) indicate that aberrant methylation is associated with negative status of the receptor.

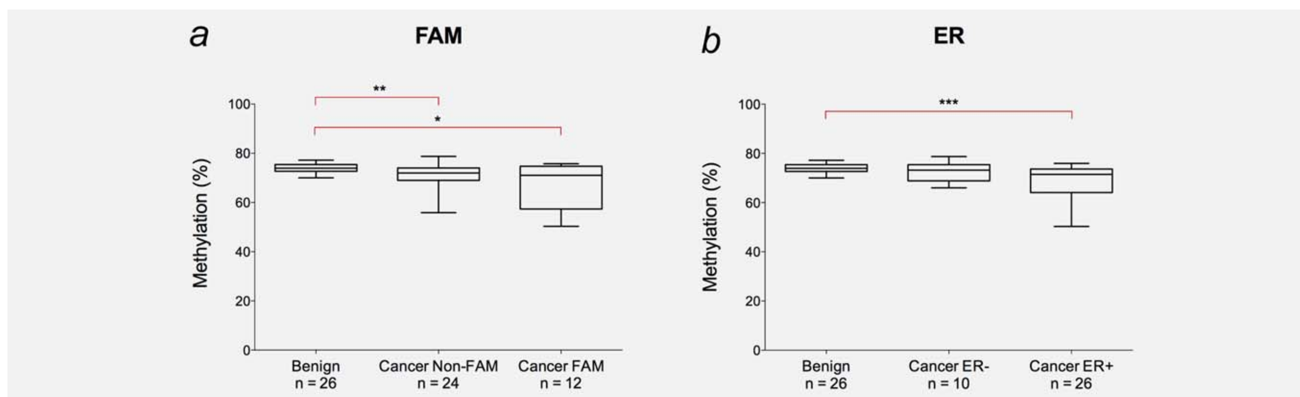

Figure 3. DNA methylation at LINE-1 elements in benign breast disease tissue and breast tumors. Results are displayed in relation to the FAM status of the tumors (*a*) and by expression of the estrogen receptor (ER) (*b*). Boxes correspond to the median and interquartile range, and the whiskers to the full range of measured values. Statistically significant differences are indicated (Mann–Whitney *U*-test; *$p < 0.05$; **$p < 0.01$ and ***$p < 0.0005$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of cancers and the expression of the ER (Figs. 3a and 3b). Greater variation was observed in breast tumors, with values ranging between 50% and 79% compared with 69–77% in benign breast disease tissue (Fig. 3a). Within the tumor samples, median values were similar in the FAM (71.1%) and non-FAM (71.8%) groups, but slightly higher in ER-negative tumors (73.2%) than in ER-positive ones (71.5%). Tumors expressing the ER also displayed a greater range of values (50–76%) in comparison with those that do not (66–79%) (Fig. 3b).

Monoallelic expression of imprinted genes is maintained in breast tumors

To determine the impact of the observed methylation changes, we examined the allele-specific expression of the genes. First, we genotyped the cancer patients for SNPs in the *DIRAS3*, *GRB10*, *H19*, *IGF2*, *MEST* and *PEG3* genes. *KCNQ1* and *SNRPN* were not analyzed because of the infrequency of aberrant methylation in the tumor samples. We identified 24 patients who were heterozygous for SNPs in the *PEG3* gene, 18 for *IGF2*, 14 for *GRB10*, 10 for *H19*, 7 for *MEST* and 4 for *DIRAS3*.

Monoallelic expression, defined here as >85% of transcripts from a single allele, was almost exclusively retained (Fig. 4). *PEG3* was monoallelically expressed in 17 of 18 patients with normal methylation levels and five of six patients displaying hypermethylation of the gene (Fig. 4a). In the other two patients, 71% and 81% of transcripts originated from a single allele. Monoallelic expression was observed in five of the seven patients informative for *MEST*, with 79% and 80% of transcripts expressed from a single allele in the other two patients (Fig. 4b). All 18 patients informative for *IGF2* monoallelically expressed the gene, including the four patients with hypomethylation of *DMR0* and four displaying hypomethylation of *DMR2* (Figs. 4c and 4d). Monoallelic expression of *H19* was observed in eight of the ten informative patients, including the one displaying hypomethylation of the gene (Fig. 4e). Two individuals displayed biallelic expression despite normal methylation profiles, with the relative expression of the two alleles being 64%/36% and 55%/45%. *GRB10* displayed a markedly different pattern of expression, with the proportional expression of the two alleles between 51%/49% and 60%/40% in seven of the 14 informative patients, and only three patients expressed >70% of transcripts from a single allele (Fig. 4f). *DIRAS3* results are not shown because of the lack of informative patients.

GRB10 and IGF2 are differentially expressed in ER-positive tumors

To further examine the relation between hormone receptor status and the expression of imprinted genes, we utilized gene expression microarray data from 302 breast tumor samples, made available through the Walter Reed Army Medical Center. Expression of seven of the imprinted genes was analyzed between ER-positive ($n = 199$) and ER-negative ($n = 103$) tumors and between PR-positive ($n = 153$) and PR-negative ($n = 149$) tumors. *H19* expression was not covered by the array. *GRB10* expression was 1.4-fold lower ($p = 1.7 \times 10^{-10}$) and *IGF2* expression was 1.9-fold higher ($p = 6.0 \times 10^{-7}$) in ER-positive tumors (Supporting Information Table 5). No significant differences were observed by PR status.

Expression of DNA methyltransferases is not associated with hormone receptor status

To investigate how DNA methylation at imprinted regions may be related to estrogen and PR status, we utilized gene expression microarray data from 302 breast tumor samples (Supporting Information Table 5). We observed no significant differences in the expression of the *DNMT1* (−1.1-fold change), *DNMT3A* (−1.3-fold change) and *DNMT3B* (−1.6-fold change) genes in ER-positive tumors in comparison with ER-negative ones. Similarly, no significant difference was observed for any of the three genes (−1.1, −1.1 and −1.3-fold changes, respectively) in PR-positive tumors *versus* PR-negative tumors.

Discussion

We found the imprinted regions *DIRAS3 DMR*, *GRB10 ICR*, *H19 ICR*, *IGF2 DMR0*, *IGF2 DMR2*, *MEST DMR* and *PEG3*

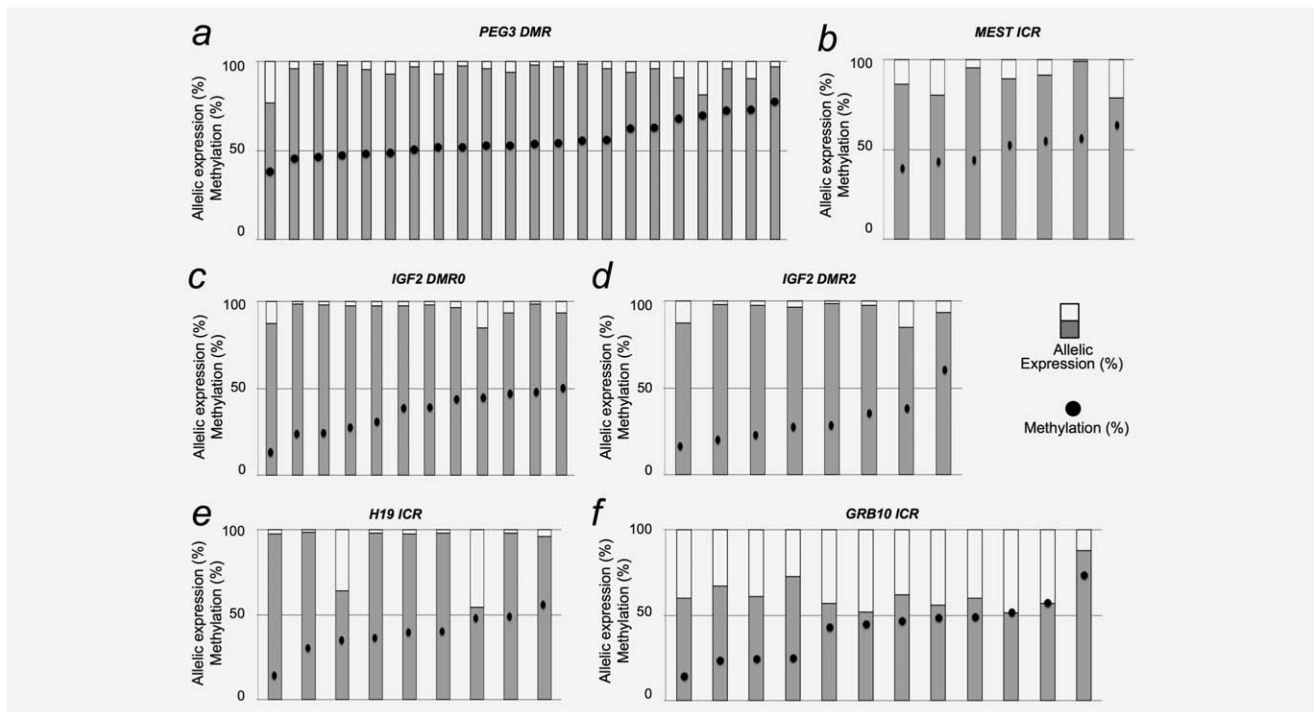

Figure 4. Expression of imprinted genes relative to DNA methylation status. Allelic-expression of *PEG3* (*a*), *MEST* (*b*) *IGF2 DMR0* (*c*) *IGF2 DMR2* (*d*), *H19* (*e*) and *GRB10* (*f*). For each patient, DNA methylation values (•) and allelic expression (bar graph) are provided. Expression values correspond to the percentage of transcripts originating from each allele.

DMR to be frequently aberrantly methylated in the patients with invasive breast cancer included in this study. These alterations are highly tissue- and tumor-specific, with no such changes observed in blood and only highly infrequently in normal breast tissue and benign breast disease tissue. This result confirmed our previous work in which we observed no correlation between the methylation at six imprinted regions in breast tumors and matched peripheral blood.[18] Variation in DNA methylation was significantly greater at all nine imprinted regions in breast tumors compared with normal breast tissue, and at eight (unadjusted model) and six (adjusted) of the regions in benign breast disease tissue. Aberrant methylation of more than three of the imprinted regions was significantly associated with negative status of the estrogen and PRs. Despite this disruption of DNA methylation, monoallelic expression of the imprinted genes was frequently maintained.

This is the first study to identify a correlation between the methylation of imprinted genes and expression of the estrogen and PRs in breast cancer. ER-positive and ER-negative tumors have distinct global DNA methylation profiles,[19,20] and works in animal models and cell lines have demonstrated that hormone receptor signaling, including that by agonists such as bisphenol A, can affect the expression of DNA methyltransferases[21–23] and directly lead to changes in the methylation and expression of imprinted genes such as *IGF2* and *PEG3*.[21,24,25] Furthermore, *in vitro* studies have shown that expression of the imprinted gene *CDKN1C* is repressed *via* epigenetic mechanisms induced by estrogen signaling in breast cancer cell lines.[26] Taken together with our observations in primary human breast tumors, this may suggest that normal hormone receptor signaling is important for the maintenance of methylation for imprinted genes, and absence of such signaling may result in aberrant regulation in malignant cells. Interestingly, *in vitro* work has demonstrated that mutations in the *DNMT1* gene lead to loss of *IGF2* and *PEG3* imprinting, with *KCNQ1* less susceptible to such changes,[27] and we similarly observed that aberrant methylation of the *IGF2* and *PEG3* DMRs was substantially more frequent than at the *KvDMR* (Fig. 2a), although we did not observe an impact upon allele-specific expression. However, we did not observe significantly different expression of *DNMT1*, *DNMT3A* or *DNMT3B* between ER-positive and ER-negative tumors (Supporting Information Table 5). Our findings may suggest that either the relation between DNA methylation at imprinted loci and ER signaling may be confined to a subset of ER-negative tumors, or the association is independent of the regulation of DNA methyltransferase expression reported *in vitro*.

Similar associations between DNA methylation and hormone receptor status have been identified for nonimprinted genes.[3,28] However, the differential methylation of alleles and roles in regulating proliferation and differentiation make imprinted genes particularly susceptible to driving tumorigenesis. Indeed, changes in the methylation of imprinted genes have been reported in esophageal dysplasia[29] and localized ovarian tumors.[30] As the ER is frequently silenced by promoter methylation in breast tumors,[31] it is not clear

whether hormone receptor status is the cause or product of wider epigenetic dysregulation. Further work is required to establish whether a subset of breast tumors may display a characteristic epigenetic profile. The identification of such a group may open new therapeutic options to the patients, including the combined use of 5-azacytidine and S-adenosylmethionine, which inhibit the growth of breast cancer cells.[32]

We observed increasing variation in DNA methylation from normal breast tissue to benign breast disease to cancer. This finding is consistent with observations made by Teschendorff *et al.*,[12] who suggested increased variability as a marker for early detection of cervical cancer, even being present in cytologically normal cells of individuals who later developed cancer. Thus, this variability may be a key part of the neoplastic transformation process. It may arise through the shifting of methylation boundaries, rather than gene-specific changes.[11] As we have used a candidate-gene approach, with comparatively short reads obtained by pyrosequencing, it remains uncertain whether this variability is confined to the interrogated DMRs or whether it may be the result of wider boundary-shifts. Furthermore, the degree of variation in methylation within the tumors remains unknown. Mosaicism is a well-known phenomenon in cancer, and may provide some explanation for our observations.

Increased variability in DNA methylation has been proposed as a blood-based marker for ovarian cancer,[33] and we similarly observed significantly increased variation in both the adjusted and unadjusted models at *GRB10 ICR*, *PEG3 DMR* and *SNRPN/SNURF ICR* in peripheral blood from cancer patients. However, the statistical significance may be the product of the tight clustering of values observed in healthy individuals, with mean deviations from median values below 3.5% at all sites. Although group differences may be significant, the value for risk prediction in individuals remains uncertain.

The sites displaying the most frequent aberrant methylation and greatest variation were the two *IGF2* DMRs. *IGF2* is a widely studied oncogene that stimulates cellular proliferation, and hypomethylation of *DMR0* is associated with poor prognosis with colorectal cancer.[34] We observed that hypomethylation of *DMR0* was associated with negative ER status, which is itself associated with poor prognosis in breast cancer.[13] The observed frequency of hypomethylation was similar to that reported elsewhere.[16] Although loss of *IGF2* imprinting in peripheral blood has been suggested as a potential diagnostic marker for colorectal cancer,[8] changes in methylation at *DMR0* were specific to the tumors, and we have previously reported a lack of correlation between blood and tumor tissues.[18] This may suggest that peripheral blood cannot serve as a surrogate tissue, although correlations may be gene-specific, as there is evidence that methylation of *ATM*[35] and targets of ER signaling[36] could be used as blood-based markers of breast cancer risk.

Interestingly, the disruption of methylation did not impact upon allele-specific expression in the tumors, with monoallelic expression almost exclusively retained. Conversely, our results suggest that *GRB10*, which is monoallelically expressed in fetal brain tissues and placenta but not in other fetal tissues,[37–39] may not be imprinted in breast tissue in adult humans. Loss of *IGF2* imprinting has been reported in colorectal and ovarian tumors[8,40] and *PEG3* in gynecologic cell lines,[41] but we observed >77% of transcripts originating from a single allele in all the tumor samples informative for these genes. Furthermore, our observation of monoallelically expressed *MEST* in all seven informative patients is in direct contrast to findings elsewhere of biallelic expression in breast cancer patients.[42] Although *H19* was biallelically expressed in two patients, methylation of the *H19 ICR* was not disrupted in these tumors, suggesting an alternative cause of LOI. Hypomethylation of *H19 ICR* has been correlated with LOI in lung cancer,[43] but there is evidence that the IGF2/H19 competition model does not hold true in colorectal[44,45] and ovarian[40] tumors. Similarly, a lack of correlation has been observed between hypomethylation of *IGF2 DMR0* and LOI in colon and breast tumors,[16] whereas hypomethylation of *IGF2 DMR0* in ovarian serous tumors increases overall expression but does not result in biallelic expression.[40] Although the classical model of imprinting suggests that monoallelic expression is the product of differential methylation of the two alleles, this does not seem to hold as strongly in humans as it does in mice. However, it is not clear why the frequency of LOI is lower here than that reported elsewhere, as this phenomenon has been reported in primary breast tumors.[42,46] An alternative explanation for our observations could be that the changes in DNA methylation are confined to a subset of cells in which expression is silenced. As pyrosequencing enables the measurement of the relative abundance of the transcripts from the two alleles, but not their overall quantity, our observations of monoallelic expression being retained may be based upon an inability to detect silencing of the expressed allele in the affected cells.

There have been few studies investigating epigenetic dysregulation in benign breast disease. Although proliferative benign disease is associated with a greater relative risk of developing cancer,[47] we have previously reported no significant difference in methylation at six of the imprinted loci between proliferative and nonproliferative conditions.[18] There is limited evidence of loss of *IGF2* imprinting in benign breast disease,[48] and we observed that hypomethylation of *IGF2 DMR2* was highly frequent. Although aberrant methylation was not observed at the other regions, there was significantly greater variability in methylation at six (adjusted model) and eight (unadjusted) sites, and increased variability in cytologically normal epithelial cells is associated with increased risk of developing neoplasia.[12] Further work is required to establish the possible role of epigenetic dysregulation in the etiology of benign breast disease.

A limitation of our study is the potential difference in the proportions of cell types between the normal, benign and tumor tissues. Laser-microdissection of the tumor tissue was

performed to isolate malignant cells, but was not conducted with normal tissue to enrich the epithelial cells. Although median DNA methylation values in normal tissue were consistently close to the expected 50% value, we cannot rule out that the variation modeling may have been affected. We also cannot exclude the possibility of copy number changes influencing measured methylation values in the samples, as insufficient quantities of DNA remained following epigenetic analyses to also perform genetic analyses. Furthermore, the number of samples in this study may also have precluded us from identifying other significant correlations between DNA methylation and tumor subtype, such as with triple-negative tumors. A particular strength of this study is the access to breast tissue from healthy individuals. There is evidence that histologically normal tissue adjacent to breast tumors can possess genomic alterations seen in the cancer,[49] and therefore it is important to use breast tissue from healthy women as controls in order to accurately identify changes associated with malignant transformation. A further strength was the availability of both DNA and RNA from blood and breast tissue that has enabled us to investigate the specificity of changes in DNA methylation and the effect upon allele-specific expression.

This is the first study, to our knowledge, to identify an association between the aberrant methylation of imprinted genes and hormone receptor status in breast cancer. We have established that (*i*) aberrant events in the DNA methylation of these imprinted regions are frequent in breast tumors, (*ii*) variation is greater in both breast tissue and in blood from patients with invasive breast cancer than in healthy individuals and (*iii*) aberrant changes in DNA methylation are associated with hormone receptor status in the tumors. Further work is required to establish whether such methylation changes are the direct result of loss of hormone receptor signaling or are the product of more widespread changes in global DNA methylation.

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