Estrogen Receptor/Progesterone Receptor-Negative Breast Cancers of Young African-American Women Have a Higher Frequency of Methylation of Multiple Genes than Those of Caucasian Women

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ABSTRACT

Purpose: To provide a molecular rationale for negative prognostic factors more prevalent in African-American (AA) than Caucasian (Cau) women, we investigated the frequency of promoter hypermethylation in invasive ductal breast cancers in the two races.

Experimental Design: HIN-1, Twist, Cyclin D2, RAR-β, and RASSFIA were analyzed in DNA from 67 AA and 44 Cau invasive ductal breast cancers, stratified by age and estrogen receptor/progesterone receptor (ER/PR) status, by methylation-specific PCR. Hierarchical multiple logistic regression analysis was applied to determine estimated probabilities of methylation. Expression of HIN-1 mRNA was analyzed by in situ hybridization and quantitative reverse transcribed PCR.

Results: Significant differences between races were observed in the ER−/PR−, age < 50 subgroup; AA tumors had higher frequency of methylation (P < 0.001) in four of five genes as compared with Cau and also a higher prevalence (80 versus 0%; P < 0.005) of three or more methylated genes per tumor. No differences in gene methylation patterns were observed across the two races for ER+/PR+ tumors in all ages and ER−/PR− tumors in age > 50. ER+/PR+ status was associated with higher frequency of methylation in Cau tumors of all ages but only with the age > 50 subgroup in AA. Frequent Cyclin D2 methylation was significantly associated (P = 0.01) with shorter survival time.

Conclusion: ER−/PR−, age < 50 tumors in AA women, have a significantly higher frequency of hypermethylation than in those of Cau women. Comparative studies, such as these, could provide a molecular basis for differences in tumor progression and pathology seen in the two races.

INTRODUCTION

Among different racial groups within the United States, overall breast cancer incidence is highest in Caucasian (Cau) women followed by African-American (AA) women (1). Compared with Cau, AA women in the <50 years age group have a higher incidence of breast cancer, whereas in the >50 age group, incidence is lower (1). However, mortality attributable to breast cancer in all age groups is higher in AA than Cau and higher than in all races (1). Negative prognostic factors for survival, which are more prevalent in AA than Cau women, are late stage at diagnosis (increased tumor size, node positive, and more distant metastasis), early onset disease (<50 years age), estrogen receptor negative (ER−) progesterone receptor negative (PR−), poorly differentiated tumors, high S phase and mitotic index, obesity, and more adverse p53 mutations (2).

There is sparse literature on the molecular basis for these clinicopathological differences in breast cancer between the two races. Unique inherited BRCA1 and BRCA2 (3) and p53 mutations (2), H-ras-1 and (4) CYP1A1 polymorphisms (2), and overexpression of Cyclin D1 (5) have been associated more strongly with AA than Cau breast cancer.

Loss of gene expression by promoter hypermethylation has been shown to have clinical implications in some cancers (6, 7). Hypermethylation-mediated loss of gene expression could provide the cell with growth-promoting characteristics, such as insensitivity to antigrowth signals, self-sufficiency in growth signals, limitless replicative potential, and evasion of apoptosis (8). HIN-1, a putative cytokine, has a role in regulating the proliferation and differentiation of normal luminal mammary epithelial cells (9). HIN-1 methylation is reported in 74% of primary breast carcinomas and ~100% of ductal carcinoma in situ (9, 10), where its expression is undetectable. Almost 40% of primary breast cancers and ~28% of in situ cancers are methylated for Twist, a gene implicated in apoptosis (8, 10, 11). Cyclin D2 is involved in cell cycle regulation. It is methylated in 50% of primary breast carcinomas and ductal carcinoma in situ, suggesting that loss of its expression is an early event in tumorigenesis (10, 12). RASSFIA, a putative tumor suppressor...
gene, is methylated in 50–60% of primary breast carcinomas (10, 13) and associated with poor survival in non-small cell lung cancer patients (13, 14). Retinoic acid receptor (RAR)-β functions in inhibition of proliferation, apoptosis, and senescence (8) and is methylated in ~50% of invasive and *in situ* breast cancers (10, 15).

Identifying race-specific molecular markers could lead to better understanding of the differences in the etiological factors contributing to the development of breast cancer between the two populations and also optimal clinical management and therapeutic intervention. As a first step toward this goal, epigenetic alterations of DNA promoter hypermethylation were analyzed in AA and Cau breast cancers in a panel of five genes frequently hypermethylated in breast cancer: (a) *HIN-1* (9); (b) *Twist* (11); (c) *Cyclin D2* (12); (d) *RAR-β* (*RAR-β* P2 promoter; Refs. 15 and 16); and (e) *RASSF1A* (13). Hypermethylation and loss of expression of these genes have been detected in invasive breast cancer but not in normal mammary epithelial cells, mammary stroma, and WBCs. Differences in DNA methylation in tumors in subgroups stratified by race, ER/PR status, and age are discussed.

**MATERIALS AND METHODS**

**Tissues.** Paraffin-embedded blocks and frozen tissues (67 AA and 44 Cau) were obtained from the Surgical Pathology Department of the Johns Hopkins Hospital and Howard University Cancer Center after approval by the human investigations committees from both institutions. All breast tumors were invasive ductal breast cancers except for two AA samples, which were mixtures of invasive ductal breast cancer and invasive lobular carcinoma.

**DNA Extraction and Methylation-Specific PCR (MSP).** DNA was extracted (10) from fixed or frozen sections (after a 30-s wash in 70% ethanol, followed by water and air drying), and MSP analysis on sodium bisulfite-treated DNA was performed as described (10, 17). The primer sequences used are described earlier (10). MSP analysis for ≥10 samples was performed in both the Hopkins and Howard University laboratories to control for any technical bias.

**mRNA analysis by Quantitative Reverse Transcribed-PCR and in Situ Hybridization.** The following primer and probe sets were used to amplify cDNA: *HIN-1*, forward, 5′CATGAAGCTGCCGCCTCT3′; reverse, 5′CTTGCGCGA-GCCACTAAG3′; probe, FAM-CCTGTGCTCTCCTGTCC-CTGCA-TAMRA; *glyceraldehyde-3-phosphate dehydrogenase*, forward, 5′CCATGTTGCTGATGGGTTG3′; reverse, 5′-TGTTGCTAGTCCCTTCCACGATA-3′; probe, TET-CTG-CACCCACACTGTTAG-TAMRA. The comparative threshold cycle method was used to analyze expression in different tissue samples (18). The experimental data were normalized to the internal *glyceraldehyde-3-phosphate dehydrogenase* control and normal breast tissue (at surgical margins of the tumors). *In situ* hybridization was performed for *HIN-1* as described previously (19).

**Statistical Analysis.** Fisher’s exact test was used to compare categorical variables across race. Standard logistic regression analyses were performed to test the association of important risk factors, *i.e.*, lymph node, stage, and grade with methylation. The Cox proportional hazards model and Log-rank test were used for assessing associations between methylation and survival outcomes and patient characteristics and survival outcomes. In this study, we consider many hypothesis tests. Therefore, we adopt a more stringent α cutoff of 0.005 for reporting significant results (*i.e.*, significance is determined by $P \leq 0.005$). To examine whether methylation patterns differ by race within subgroups defined by age and ER/PR status, we used a multilevel model, similar to a multiple logistic regression but fits the logistic model to all genes simultaneously, with the assumption that effects across genes are from a common distribution. Motivation (20–22) and implementation details (23) are given in the supplementary materials.

**RESULTS**

MSP analysis was performed on DNA from 67 AA and 44 Cau primary invasive ductal breast cancer (Table 1; Fig. 1). Comparative prevalence of promoter hypermethylation of *HIN-1, Twist, Cyclin D2, RAR-β*, and *RASSF1A* genes and methylation of multiple (more than or equal to three) genes in AA and Cau breast carcinomas as stratified by ER/PR status and age are shown in Table 2.

We used multiple logistic regression analysis to assess the association of risk factors, such as lymph node status, tumor grade, and stage with methylation of the five genes. None of the risk factors showed significant association with methylation of

<table>
<thead>
<tr>
<th>Table 1 Patient and tumor characteristics</th>
<th>AA*</th>
<th>Cau</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (yrs)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;50</td>
<td>38 (57)</td>
<td>23 (52)</td>
<td>0.70</td>
</tr>
<tr>
<td>&lt;50</td>
<td>29 (43)</td>
<td>21 (48)</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>ER/PR status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>30 (45)</td>
<td>23 (52)</td>
<td>0.56</td>
</tr>
<tr>
<td>Negative</td>
<td>37 (55)</td>
<td>21 (48)</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>Elston grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2 (3)</td>
<td>3 (7)</td>
<td>0.54</td>
</tr>
<tr>
<td>II</td>
<td>30 (45)</td>
<td>17 (39)</td>
<td>0.40</td>
</tr>
<tr>
<td>III</td>
<td>35 (52)</td>
<td>24 (55)</td>
<td>0.046</td>
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<td><strong>Lymph node status</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>33 (49)</td>
<td>32 (73)</td>
<td>0.046</td>
</tr>
<tr>
<td>Negative</td>
<td>29 (43)</td>
<td>12 (27)</td>
<td>0.046</td>
</tr>
<tr>
<td>Unknown</td>
<td>5 (7)</td>
<td>0 (0)</td>
<td>0.046</td>
</tr>
</tbody>
</table>

*AA*, African-American; *Cau*, Caucasian; *ER*, estrogen receptor; PR, progesterone receptor.

*P* determined by Fisher’s exact test.

7 http://cancerres.aacrjournals.org.
any gene (data not shown) except for Twist methylation, which showed a borderline association with stage 2/3 of breast cancer ($P = 0.03$). To assess the association between age (age $< 50$ versus $> 50$), race (AA versus Cau), and ER/PR status (positive versus negative) and their interactions, a multilevel, multivariate logistic regression model showed that the main effect of age < 50, main effect of race, two-way interaction between race and ER/PR, and two-way interaction between age $< 50$ and ER/PR were constant across genes. The model showed that the following effects varied across genes: (a) the main effects of ER/PR; (b) two-way interaction between race and age $< 50$; and (c) three-way interaction between race, age $< 50$, and ER/PR. The results from the model are shown as estimated percentage of methylation for each gene and methylation of multiple (more than or equal to three) genes in Fig. 2.

**Major Differences between AA and Cau Breast Cancers Are in the Age $< 50$, ER$^-$/PR$^-$ Subgroup.** ER$^+$/PR$^+$ tumors in all age subgroups (age $> 50$ and $< 50$) showed essentially no difference in estimated percentage of methylation across race for any of the five genes: (a) HIN-1; (b) Twist; (c) Cyclin D2; (d) RAR-$\beta$; and (e) RASSF1A (Fig. 2).

We found pronounced differences in an estimated percentage of frequency of methylation across race in the ER$^-$/PR$^-$, age $< 50$ subgroup (Fig. 2). Breast tumors in ER$^-$/PR$^-$, age $< 50$ AA women, had a significantly higher estimated percentage of methylation for four of five genes, as compared with those from Cau women in this subgroup. The estimated percentages of frequency of methylation were: (a) HIN-1, 79% in AA versus 19% in Cau ($P < 0.0001$); (b) Twist, 67% in AA versus 16% in Cau ($P < 0.0001$); (c) Cyclin D2, 64% in AA versus 19% in Cau ($P < 0.0001$); and (d) RASSF1A, 76% in AA versus 29% in Cau ($P < 0.0001$). We observed a similar trend for RAR-$\beta$ with an estimated frequency percentage of methylation of 40% in AA versus 8% in Cau, although this difference was not statistically significant ($P = 0.01$). On the contrary, no race-related effects on methylation were observed in the ER$^+$/PR$^+$, age $> 50$ subgroup of tumors (Fig. 2).

**Methylation As a Function of ER/PR Status and Age within Each Race.** Looking at an estimated percentage of methylation patterns within the same race (Fig. 2), we observed that in the age $> 50$ subgroup, both Cau and AA tumors had significantly higher methylation for HIN-1 and RASSF1A in the ER$^+$/PR$^+$ versus ER$^-$/PR$^-$ tumors [Cau: HIN-1, 85 versus 38% ($P < 0.0001$); RASSF1A, 92 versus 51% ($P < 0.0001$); AA: HIN-1, 87 versus 50% ($P < 0.0001$); RASSF1A, 93 versus 63% ($P < 0.0001$)]. In the age $< 50$ subgroup, Cau tumors followed the same trend as seen above, and this effect was consistent in four of five genes [ER$^+$/PR$^+$ versus ER$^-$/PR$^-$: HIN-1, 84 versus 19% ($P < 0.0001$); Cyclin D2, 52 versus 19% ($P < 0.005$); RAR-$\beta$, 37 versus 8% ($P < 0.005$); RASSF1A, 91 versus 29% ($P < 0.0001$)]. The Twist gene showed a similar trend (44% in ER$^+$/PR$^+$ versus 16% in ER$^-$/PR$^-$), but this difference was not significant ($P = 0.02$). Surprisingly, AA tumors in the age $< 50$ subgroup did not show the same trend of higher methylation in ER$^+$/PR$^+$ tumors that was seen in the other subgroups. As evident in Fig. 2, ER$^-$/PR$^-$ age $< 50$ tumors from AA women clustered with ER$^+$/PR$^+$ subgroups in all comparisons.

**Methylation at Multiple Loci per Tumor.** Using the gene-specific estimate described thus far, we also estimated prevalence of multiple (more than or equal to three) gene methylation in each subgroup (Fig. 2). All ER$^+$/PR$^+$ tumors

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**Table 2** Frequency of DNA methylation in AA$^a$ and Cau breast carcinomas as stratified by ER/PR status and age

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>HIN-1</th>
<th>Twist</th>
<th>Cyclin D2</th>
<th>RAR-$\beta$</th>
<th>RASSF1A</th>
<th>$\geq 3$ genes/tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cau</td>
<td>24/44 (55%)</td>
<td>15/44 (34%)</td>
<td>18/44 (41%)</td>
<td>11/42 (26%)</td>
<td>32/44 (73%)</td>
<td>19/44 (43%)</td>
</tr>
<tr>
<td>AA</td>
<td>50/66 (81%)</td>
<td>33/64 (52%)</td>
<td>36/67 (54%)</td>
<td>22/65 (34%)</td>
<td>52/67 (78%)</td>
<td>42/67 (63%)</td>
</tr>
<tr>
<td>ER$^-$/PR$^-$ &gt; 50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cau</td>
<td>3/11 (27%)</td>
<td>3/11 (27%)</td>
<td>3/11 (27%)</td>
<td>4/9 (44%)</td>
<td>7/11 (64%)</td>
<td>3/11 (27%)</td>
</tr>
<tr>
<td>AA</td>
<td>9/22 (41%)</td>
<td>8/20 (40%)</td>
<td>13/22 (59%)</td>
<td>5/20 (25%)</td>
<td>15/22 (68%)</td>
<td>10/22 (45%)</td>
</tr>
<tr>
<td>ER$^-$/PR$^-$ &lt; 50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cau</td>
<td>2/10 (20%)</td>
<td>2/10 (20%)</td>
<td>3/10 (30%)</td>
<td>0/10 (0%)</td>
<td>2/10 (20%)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>AA</td>
<td>14/15 (93%)</td>
<td>12/15 (80%)</td>
<td>9/15 (60%)</td>
<td>3/15 (20%)</td>
<td>11/15 (73%)</td>
<td>12/15 (80%)</td>
</tr>
<tr>
<td>ER$^+$/PR$^+$ &gt; 50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cau</td>
<td>10/12 (83%)</td>
<td>7/12 (58%)</td>
<td>5/12 (42%)</td>
<td>3/12 (25%)</td>
<td>12/12 (100%)</td>
<td>8/12 (67%)</td>
</tr>
<tr>
<td>AA</td>
<td>15/16 (94%)</td>
<td>8/15 (53%)</td>
<td>9/16 (56%)</td>
<td>6/16 (38%)</td>
<td>14/16 (88%)</td>
<td>12/16 (75%)</td>
</tr>
<tr>
<td>ER$^+$/PR$^+$ &lt; 50</td>
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</tr>
<tr>
<td>Cau</td>
<td>9/11 (82%)</td>
<td>3/11 (27%)</td>
<td>7/11 (64%)</td>
<td>4/11 (36%)</td>
<td>11/11 (100%)</td>
<td>8/11 (73%)</td>
</tr>
<tr>
<td>AA</td>
<td>12/13 (92%)</td>
<td>5/14 (36%)</td>
<td>5/14 (36%)</td>
<td>8/14 (57%)</td>
<td>12/14 (86%)</td>
<td>8/14 (57%)</td>
</tr>
</tbody>
</table>

$^a$ AA, African-American; Cau, Caucasian; ER, estrogen receptor; PR, progestosterone receptor.

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**Fig. 1** Methylation-specific PCR analysis on each sample using unmethylated (U) and methylated (M) sequence-specific primers. DNA from WBC’s (−) and MDA-MB-231 (+) breast cancer cell line served as negative and positive controls for methylated genes, respectively. Water (W) as negative control for both unmethylated and methylated genes. WBC also served as a positive control for unmethylated genes.
had a higher prevalence of methylation at multiple loci. Across races, the most striking difference was again seen in the ER−/PR− age ≤ 50 subgroup of tumors. AA tumors in this group had a very high proportion of tumors (estimated 80%) with multiple methylated genes as compared with Cau tumors (estimated 0%; \( P < 0.005 \)). We computed odds ratios for the association between methylation of two genes and found highly significant associations between \( \text{HIN-1} \) and \( \text{RASSF1A} \), \( \text{HIN-1} \) and \( \text{Twist} \), and \( \text{Cyclin D2} \) and \( \text{Twist} \) methylation (data not shown). In addition, we noted that all pairwise odds ratios showed a positive association between methylation events, although not significant.

**Hypermethylation of \( \text{HIN-1} \) Correlates with mRNA Expression in the Tumors.** To assess the biological significance of hypermethylation in this study, as a representative, we analyzed the expression of \( \text{HIN-1} \) in primary tumors using quantitative reverse transcribed PCR. We analyzed 12 tumors that contained an unmethylated and 14 tumors that contained a methylated \( \text{HIN-1} \) gene. As predicted, compared with normal breast tissue, 10 of 14 tumors (71%) methylated for \( \text{HIN-1} \) gene showed no detectable expression of \( \text{HIN-1} \) mRNA (Fig. 3A). Of the remaining 4, 3 showed very low levels, and only 1 tumor, contrary to expectation, showed a high level of expression. Seven of 12 tumors (58%), unmethylated for \( \text{HIN-1} \), showed detectable mRNA expression, whereas 5 did not. Not observing expression in all of the tumors in this group is consistent with previous findings that \( \text{HIN-1} \) expression is silenced in breast cancer, primarily by methylation of its promoter sequences, but other modes of transcriptional silencing are operative in tumors containing unmethylated \( \text{HIN-1} \) genes (9).

We further confirmed the results of the quantitative reverse transcribed PCR analysis of \( \text{HIN-1} \) expression by performing in situ hybridization on paraffin-embedded tissue sections. Six tumors unmethylated and five methylated for the \( \text{HIN-1} \) gene were analyzed by in situ hybridization (representative photomicrographs shown in Fig. 3B). Five of six (83%) unmethylated tumors were positive for \( \text{HIN-1} \) expression, whereas only two (40%) of five methylated tumors showed partial expression of \( \text{HIN-1} \) (<30% cells). Thus, using two independent approaches, we observed a direct correlation between \( \text{HIN-1} \) methylation and loss of mRNA expression.

**Correlating Methylation with Clinical Outcome of Patients.** We analyzed patient data to investigate whether frequency of methylation was associated with time of diagnosis to time to all cause death or time to cancer death (data not shown). We view these analyses as exploratory because: (a) our sample size was small; and (b) the presence of a potential confounding factor that the patients received a variety of treatments. In this data set, we found that tumor grade III and ER/PR status were associated with survival. We performed...
Cox proportional hazards model analyses, where tumor grade and ER/PR status were included as covariates. There was evidence of a significant association of cancer-related death (Hazard’s ratio = 3.82, P = 0.01) with highly frequent methylation of Cyclin D2 alone. Interestingly, in accord with these findings, multiple logistic regression analyses also showed that the estimated percentage of methylation of Cyclin D2 gene was significantly (P < 0.0001) more prevalent in AA (64%) than Cau (19%) breast cancers in the age < 50, ER−/PR− subgroup of breast tumors (Fig. 2).

**DISCUSSION**

Survival of breast cancer patients is significantly worse in AA than Cau women for reasons not well understood at the molecular level. Hormone-negative receptors and early age of onset are negative prognostic factors that are more prevalent in AA than in Cau breast cancers (2). In this study, we investigated the hypothesis that breast cancers in AA and Cau women are characterized by many common but some distinct molecular alterations that result in altered patterns of gene expression and differences in clinical presentation and behavior. The more aggressive tumors and early onset breast cancer in AA women may be caused by specific alterations in gene expression patterns that are different or more pronounced in AA than in Cau. ER/PR status has been associated with methylation of BRCAI, the ER-α gene, and HIN-1. In breast cancer, we did not see an association of methylation with age or any of the other risk factors, such as lymph node status, tumor grade, and stage (data not shown). This suggests that, as demonstrated in colon cancer...
by Yamashita et al. (33), one could question the existence of a methylator phenotype in breast cancer.

Our study was limited by the sample size to make any correlation of methylation with survival in the different subgroups. However, we did observe an association of Cyclin D2 methylation with survival. Cyclin D2 was significantly more methylated in ER−/PR− AA tumors as compared with their Cau counterparts.

In conclusion, by analyzing the hypermethylation profiles of five genes, we could decipher many significant disparities and some similarities between AA and Cau breast cancers. In the ER−/PR− subgroup, higher methylation in AA breast cancers compared with Cau breast cancers is associated with negative prognostic factors (ER status and age) that are more prevalent in AA. This observed heterogeneity between AA and Cau breast cancers underscores the need for a more comprehensive comparison of breast cancers between these races, which could lead to better clinical management for different racial groups.

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REFERENCES

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