

Racial Variation in Breast Tumor Promoter Methylation in the Carolina Breast Cancer Study

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Abstract

Background: African American (AA) women are diagnosed with more advanced breast cancers and have worse survival than white women, but a comprehensive understanding of the basis for this disparity remains unclear. Analysis of DNA methylation, an epigenetic mechanism that can regulate gene expression, could help to explain racial differences in breast tumor clinical biology and outcomes.

Methods: DNA methylation was evaluated at 1,287 CpGs in the promoters of cancer-related genes in 517 breast tumors of AA ($n = 216$) or non-AA ($n = 301$) cases in the Carolina Breast Cancer Study (CBCS).

Results: Multivariable linear regression analysis of all tumors, controlling for age, menopausal status, stage, intrinsic subtype, and multiple comparisons [false discovery rate (FDR)], identified seven CpG probes that showed significant (adjusted $P < 0.05$) differential methylation between AAs and non-AAs. Stratified

analyses detected an additional four CpG probes differing by race within hormone receptor–negative (HR[−]) tumors. Genes differentially methylated by race included *DSC2*, *KCNK4*, *GSTM1*, *AXL*, *DNAJC15*, *HBII-52*, *TUSC3*, and *TES*; the methylation state of several of these genes may be associated with worse survival in AAs. TCGA breast tumor data confirmed the differential methylation by race and negative correlations with expression for most of these genes. Several loci also showed racial differences in methylation in peripheral blood leukocytes (PBL) from CBCS cases, indicating that these variations were not necessarily tumor-specific.

Conclusions: Racial differences in the methylation of cancer-related genes are detectable in both tumors and PBLs from breast cancer cases.

Impact: Epigenetic variation could contribute to differences in breast tumor development and outcomes between AAs and non-AAs. *Cancer Epidemiol Biomarkers Prev*, 24(6): 921–30. ©2015 AACR.

Introduction

Breast cancer, the most common cancer among women in the United States (1), is a heterogeneous disease with multiple clinical, histopathologic, and molecular subtypes, exhibiting different therapeutic responses and prognoses (2–4). Racial differences exist in its presentation and outcome, with African-American (AA) women, especially younger women, being diagnosed with more advanced cancers (5) and having worse survival than white women even after controlling for known prognostic factors or treatment (5–7). Prior work using protein markers to classify breast tumor intrinsic subtypes in the Carolina Breast Cancer Study (CBCS) found a higher prevalence of basal-like breast

tumors among AA women compared with white women (3); however, AA women also have worse outcomes across all intrinsic subtypes (8).

In an effort to better understand the molecular factors contributing to breast cancer development, outcomes, and racial disparities, we evaluated DNA promoter methylation profiles in invasive breast tumors from the CBCS. An epigenetic modification to DNA that does not alter the nucleotide sequence (9), DNA methylation usually occurs as the addition of a methyl group to a cytosine within a CpG dinucleotide. Aberrant hypermethylation of CpG islands in tumor-suppressor genes can result in their silencing in cancer, while hypomethylation can lead to increased oncogene expression (9, 10). Such methylation changes and their effects on gene expression have the potential to influence breast tumor phenotypes and clinical outcomes (10). Several prior studies using targeted methylation approaches for a few genes have suggested that racial variation exists in methylation between AAs and non-AAs (or Caucasians; refs. 11–14), and a recent whole-genome study using the Illumina 450K platform supports these earlier reports (15).

In this study, use of a cancer-focused promoter methylation array to assess methylation in a large population-based series of breast tumors from AA and non-AA breast cancer cases in the CBCS revealed differential tumor methylation of several genes, and most of these were also differentially methylated in white blood cells, thereby enabling a more informed interpretation of which epigenetic differences might potentially contribute to breast cancer development, influence breast tumor chemosensitivity or the disparity in outcomes.

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

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doi: 10.1158/1055-9965.EPI-14-1228

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Materials and Methods

CBCS cases and specimens

The CBCS is a population-based, case–control study of incident invasive breast cancer in North Carolina. Details of the study design have been described previously (16). Randomized recruitment was used to oversample younger and AA cases to ensure that they comprised roughly half the study sample. Race/ethnicity was self-reported. Among non-AA cases, 97% self-reported as Caucasian ($n = 291$) and the other 3% included 4 cases with Hispanic ethnicity, 3 American Indians, 6 Asian/Native Islanders, and 1 other; therefore, we refer to this group as non-AA.

Blood samples were obtained for DNA extraction from peripheral blood leukocytes (PBL). Ancestry informative markers (AIM) to estimate the proportion of African versus European ancestry were previously evaluated from PBL DNA in the CBCS (17), AIMs genotypes were derived from 144 single-nucleotide polymorphisms (SNP), and an AIMs score ranging from 0 to 1.0 was generated for each case representing lower to higher African ancestry.

Formalin-fixed paraffin-embedded (FFPE) breast tumors were sectioned and mounted on slides, histopathologically reviewed, and tumor areas macrodissected for DNA extraction (18). Clinical data were obtained from medical records or histopathologic review of tumor tissue. Breast tumor intrinsic subtypes were identified using estrogen receptor (ER), progesterone receptor (PR), HER2, CK5, CK6, and EGFR protein markers (3). Hormone receptor (HR) positivity was considered to be ER⁺ and/or PR⁺, while tumors designated HR-negative (HR⁻) were both ER⁻ and PR⁻.

Methylation analysis

DNA lysates prepared from breast tumors were sodium bisulfite-treated using the EZ DNA Methylation Gold Kit (Zymo Research). Methylation profiling was accomplished using the Illumina GoldenGate Cancer Panel I array (Illumina) on 517 breast tumors and 69 PBLs from cases, 61 of which were matched to individual cases. Methylation data were preprocessed using GenomeStudio Methylation software (Illumina). Methylation is represented by β , an estimate of the fraction of methylated DNA, and ranges from 0 (unmethylated) to 1.0 (fully methylated). The array interrogated 1,505 CpG sites located in the upstream regulatory region (promoter or exon 1) of 807 genes. Of the 1,505 CpG probes, a total of 1,287 CpGs were included in the final dataset after filtering (18), including probes previously identified as overlapping an underlying sequence variant (19). Compared with 163 cases not evaluated due to inadequate quantity or quality of DNA (of 680 total with tumors in CBCS phase I), the 517 breast cancer cases evaluated were younger ($P = 0.03$) but did not differ in other characteristics. Array data were deposited to Gene Expression Omnibus under accession number GSE51557.

Statistical analyses

Statistical analyses were carried out using the R package (<http://www.r-project.org/>) or SAS 9.3. CpGs differentially methylated between breast tumors of AAs and non-AAs were identified via generalized linear models (GLM), modeling the methylation value with logit link function, and including race (AA vs. non-AA) as a predictor, while also adjusting for age, menopausal status, stage, and intrinsic subtype (20). P values were adjusted for multiple comparisons via the Benjamini–Hochberg method for controlling the false discovery rate (FDR). A GLM model to compare methyl-

ation by race was performed first for all breast tumors, and then was repeated with stratification of breast tumors on HR status. The box and whisker plots were constructed to display the distribution of methylation β values for each group, and included the mean, median, and interquartile range of methylation values, and the group minimum and maximum values. Univariate analysis of methylation by race in PBLs was determined by the Student t test. Correlation of methylation in paired tumors and PBLs from cases was determined by the Spearman rank-correlation coefficient. The Kaplan–Meier plots and the log-rank P values were used to illustrate disease-specific survival according to methylation level (high, intermediate, and low) for select probes within the AA or non-AA case groups. For each probe, cutoff points were based on the tertiles in the combined AA/non-AA dataset.

Validation studies

Independent validation of racial differences in breast tumor methylation and correlations of methylation with gene expression were conducted using breast tumor data from The Cancer Genome Atlas (TCGA; ref. 21). Methylation analysis in TCGA was performed using the Illumina Infinium 450K methylation array, while gene expression data were generated using RNA sequencing. Only 371 of the 1,505 CpG probes interrogated on the Illumina GoldenGate methylation platform exactly match those on the 450K methylation array; however, prior analysis of 450K probes showed that methylation levels were concordant for matched probes between distinct methylation platforms (22). Therefore, we compared methylation between AA and white patients at probes from the 450K array in TCGA that interrogated direct match CpG sites when available, or those closest within 200 bp upstream or downstream of the GoldenGate probes of interest. We also tested associations between methylation and gene expression for these probes. Two separate subsets of TCGA data were used for these analyses. For comparisons of breast tumor methylation, β values for each probe of interest from the 450K array were compared between 42 AA women and 291 white women in the TCGA dataset. T tests were used to identify probes that differed by race at $P < 0.05$. TCGA data from 581 breast cancer patients were used to examine relationships between methylation and gene expression. Pearson correlation coefficients were calculated in all 581 TCGA breast tumors, or within HR⁺ or HR⁻ subsets, based on RNAseq (Illumina) log₂ RSEM gene normalized expression values with methylation β values for 450K CpG probes, with significance set at $P < 0.05$. Independent validation of racial differences in methylation was also performed using an Illumina 450K methylation dataset in Gene Expression Omnibus on lymphoblastoid cell lines from healthy female AA ($n = 80$) and Caucasian American subjects ($n = 49$; accession #GSE36369; ref. 23). Moreover, technical validation was conducted to compare gene methylation from the GoldenGate array with that from quantitative methylation-specific polymerase chain reaction (Q-MSP; ref. 24).

Results

Characteristics of breast cancer cases

Characteristics of AA and non-AA cases and their tumors evaluated for DNA methylation are detailed in Table 1. Breast cancer cases were mostly early stage (>86% stages I or II). AA cases were older, more frequently postmenopausal, and more likely to

have HR⁻, high-grade, or basal-like breast tumors compared with non-AAs, as previously reported (3). PBL samples derived from a subset of AA and non-AA cases did not differ in age ($P = 0.44$) or menopausal status ($P = 0.12$).

Racial differences in breast tumor methylation

Comparison of β values for 1,287 CpG probes in 517 breast tumors using GLM while controlling for age, menopausal status, stage, intrinsic subtype, and multiple comparisons (FDR) identified a total of 24 CpG probes that differed significantly at $q < 0.05$ between AA ($n = 216$) and non-AA ($n = 301$) cases (Table 2). The majority of these probes ($n = 17$), however, were considered technically ambiguous or unreliable because they were previously reported to overlap a site of a potential SNP or repeat (19), or based on review of updated probe target sequence information in NCBI databases (e.g., Ensembl, dbSNP, and Blast) were subsequently found to overlap underlying sequence variants or show ancestral population differences of $>10\%$ (as detailed in Supplementary Table S1). In total, seven probes showing differential methylation by race were retained for further analysis, including

four that had no known underlying variant (DSC2_E90_F, KCNK4_E3_F, GSTM1_P266_F, and HBII-52_E142_F) and three that overlapped a single SNP but that was unlikely to impact probe performance due to its location toward the end of the target sequence and the lack of evidence for ancestral differences in allele frequencies (AXL_P223_R, DNAJC15_E26_R, and TES_P182_F; Table 2). Although the *GSTM1* gene is polymorphic for a deletion variant, this deletion occurs within the coding sequence and does not involve the probe target region in the promoter (25). Importantly, racial variation in methylation for these seven loci was unlikely to be appreciably related to the known racial differences in breast tumor subtype distribution or differences in methylation patterns between the major intrinsic subtypes because we adjusted for IHC-based subtype, stage, age, and menopausal status in the GLM. Racial variation in breast tumor methylation was evident for these top CpG probes whether race was self-reported or defined by AIMS (Supplementary Fig. S1).

Stratified GLM was subsequently performed to determine whether the racial differences initially observed in CpG methylation were evident within HR-defined subtypes. As shown in

Table 1. Demographic, tumor, and clinical characteristics of the AA and non-AA breast cancer cases evaluated for DNA methylation

Characteristic	AA n (%)	Non-AA n (%)	P ^a
Cases with tumors			
Total	216	301	
Age, y			0.01
50+ y	97 (44.9)	102 (33.9)	
<50 y	119 (55.1)	199 (66.1)	
Menopausal status			0.03
Postmenopausal	113 (52.3)	129 (42.9)	
Premenopausal	103 (47.7)	172 (57.1)	
Stage ^b			0.64
I	69 (34.5)	109 (38.8)	
II	104 (52.0)	141 (50.2)	
III	22 (11.0)	23 (8.2)	
IV	5 (2.5)	8 (2.8)	
Primary tumor size, cm			0.23
≤2	98 (47.1)	152 (52.6)	
>2	110 (52.9)	137 (47.4)	
Lymph node status			0.27
Negative	115 (55.6)	176 (60.5)	
Positive	92 (44.4)	115 (39.5)	
HR expression			0.01
ER ⁺ /PR ⁺	88 (42.5)	162 (55.3)	
ER ⁺ /PR ⁻	20 (9.7)	28 (9.5)	
ER ⁻ /PR ⁺	15 (7.2)	24 (8.2)	
ER ⁻ /PR ⁻	84 (40.6)	79 (27.0)	
Intrinsic subtype ^c			0.04
Luminal A	81 (47.9)	131 (53.7)	
Luminal B	22 (13.0)	43 (17.6)	
HER2 ⁺ /HR ⁻	13 (7.7)	13 (5.3)	
Basal-like	46 (27.2)	40 (16.4)	
Unclassified	7 (4.1)	17 (7.0)	
Cases with PBLs			
Total N	29	40	
Age, y			0.44
50+	9 (31.0)	16 (40.0)	
<50	20 (69.0)	24 (60.0)	
Menopausal status			0.12
Postmenopausal	9 (31.0)	20 (50.0)	
Premenopausal	20 (69.0)	20 (50.0)	

^aChi-square P values.

^bAccording to the AJCC breast tumor staging guidelines.

^cIntrinsic subtypes determined by a panel of immunohistochemical markers included luminal A (ER⁺ and/or PR⁺, HER2⁻), luminal B (ER⁺ and/or PR⁺, HER2⁺), basal-like (ER⁻, PR⁻, HER2⁻, cytokeratins CK5⁺ and/or CK6⁺, or epidermal growth factor receptor-positive), HER2⁺ (ER⁻, PR⁻, HER2⁺), and unclassified (all markers negative).

Table 2. CpG probes differentially methylated by race in multivariable GLM analysis of all breast tumors

Gene/probe	CpG ID	Gene description	Mean β^a AA (n = 216)	Mean β^a non-AA (n = 301)	Delta β	Mean β normal breast (n = 9)	Coef ^b	Age-only-adjusted g value ^c	Fully adjusted P value ^d	Fully adjusted g value ^e	Variants	Chr
No overlapping variant ^f												
DSC2_E90_F	cg08156793	Desmocollin 2	0.3564	0.2666	0.0898	0.1547	0.3777	6.48E-07	1.80E-06	3.31E-04	None	18
KCNK4_E3_F	cg01352108	Potassium channel, subfamily K, member 4	0.4318	0.3502	0.0816	0.2877	0.2644	4.07E-06	1.27E-04	9.60E-03	None	11
GSTM1_P266_F ^g	cg19763514	Glutathione S-transferase mu 1	0.5757	0.6269	-0.0512	0.4544	-0.2681	3.27E-02	3.23E-04	2.08E-02	None	1
HB11-52_E142_F	cg24501180	SNORD115-1/small nucleolar RNA, C/D box 115-1	0.4026	0.5402	-0.1376	0.5768	-0.5283	4.97E-12	3.30E-10	1.06E-07	None	15
Overlaps variant but minimal impact expected ^h												
AXL_P223_R	cg09524393	AXL receptor tyrosine kinase	0.3311	0.2489	0.0822	0.1055	0.3015	6.15E-06	5.32E-04	3.11E-02	SNP	19
DNAJC15_E26_R	cg10157207	DnaJ (Hsp40) homolog, subfamily C, member 15	0.1399	0.1938	-0.0539	0.1303	-0.3789	2.30E-05	1.57E-05	1.68E-03	SNP	13
TES_P182_F	cg00626984	Testis-derived transcript (3 LIM domains)	0.2223	0.2553	-0.0330	0.1462	-0.2989	8.61E-02	6.26E-04	3.50E-02	SNP	7
Overlaps variant and/or ancestral allelic difference ⁱ												
CCL3_P543_R	cg05481196	Chemokine (C-C motif) ligand 3	0.8906	0.8598	0.0308	0.9081	0.3098	9.80E-03	3.76E-04	2.50E-02	SNP	17
MAP2K6_E297_F	cg09190049	Mitogen-activated protein kinase kinase 6	0.1651	0.1160	0.0491	0.1085	0.3967	2.30E-05	2.24E-03	2.24E-03	SNP	17
DNAJC15_P65_F	cg05035143	DnaJ (Hsp40) homolog, subfamily C, member 15	0.8318	0.8682	-0.0364	0.8381	-0.2449	2.49E-04	9.20E-04	4.94E-02	SNP	13
ID1_P659_R	cg09569033	Inhibitor of DNA-binding 1, dominant-negative helix-loop-helix protein	0.1850	0.1336	0.0514	0.1040	0.4381	5.33E-04	1.68E-03	1.68E-03	SNP	20
PADI4_PT158_R	cg19159961	Peptidyl arginine deiminase, type IV	0.4753	0.3589	0.1164	0.4671	0.3442	7.89E-11	9.24E-06	1.32E-03	SNP	1
NTRK1_E74_F	cg18744444	Neurotrophic tyrosine kinase, receptor, type 1	0.7743	0.6043	0.1700	0.7435	0.7792	2.08E-05	1.52E-05	1.68E-03	SNP	1
NNAT_P544_R	cg10288563	Neurostatin	0.8504	0.8836	-0.0332	0.8716	-0.4324	3.24E-02	1.82E-05	1.80E-03	SNP	20
WTT_P853_F	cg08219028	Wilms tumor 1	0.1816	0.2852	-0.1036	0.1518	-0.5808	6.06E-05	2.96E-05	2.54E-03	SNP	11
HLA-DQA2_P282_R	cg09782137	Major histocompatibility complex, class II, DQ alpha 2	0.7065	0.7625	-0.0560	0.7280	-0.3296	4.73E-03	9.38E-05	7.55E-03	SNP	6
ABCB4_P892_F	cg02810586	ATP-binding cassette, sub-family B (MDR/TAP), member 4	0.6734	0.8491	-0.1757	0.9052	-0.9207	1.14E-15	5.33E-13	2.29E-10	R, SNP	7
MSH3_P13_R	cg06210628	mutS homolog 3 (<i>E. coli</i>)	0.3998	0.6643	-0.2645	0.5660	-1.1511	1.75E-30	1.32E-27	1.70E-24	Indel	5
MSH3_E3_F	cg14636131	mutS homolog 3 (<i>E. coli</i>)	0.5338	0.8121	-0.2783	0.7250	-1.4138	2.89E-27	5.40E-22	5.40E-22	Indel	9
NOTCH1_P198_F	cg26924342	Notch 1	0.1761	0.1342	0.0419	0.1536	0.3496	1.32E-05	1.05E-06	2.71E-04	R	5
ERCC1_P440_R	cg13282827	Excision repair cross-complementing rodent repair deficiency, complementation group 1	0.1702	0.1237	0.0465	0.0817	0.3462	7.14E-07	1.80E-06	3.31E-04	R	19
PTPRE_E178_R	cg09322748	Protein tyrosine phosphatase, receptor type, F	0.1429	0.1889	-0.0460	0.1073	-0.3715	1.71E-04	6.22E-06	1.00E-03	R	1
ELL_P693_F	cg09597048	Elongation factor RNA polymerase II	0.3922	0.3117	0.0805	0.2612	0.2937	2.34E-05	1.95E-04	1.39E-02	R	19
GML_E144_F	cg21475536	Glycosylphosphatidylinositol anchored molecule like	0.7043	0.7929	-0.0886	0.7469	-0.4365	3.63E-04	2.84E-04	1.92E-02	Indel	8

Multivariable GLM was conducted using 1,287 CpG probes from the Illumina Cancer Panel I array in 517 breast tumors.

^aMean of β values, which ranged from 0 (completely unmethylated) to 1 (fully methylated).

^bCoefficients for comparison of AA cases to non-AA cases; positive coefficients indicate higher methylation in AA cases compared with non-AA cases, while negative values indicate higher methylation in non-AA cases than AA cases.

^cAdjusted for age and multiple comparisons (FDR).

^dAdjusted for age, menopausal status, stage, and intrinsic subtype.

^eA adjusted for age, menopausal status, stage, intrinsic subtype, and multiple comparisons (Benjamini-Hochberg FDR).

^fNo known variant overlaps CpG probe based on review of Ensembl, dbSNP, or as annotated in Byun et al. (19).

^gGSTM1 variant occurring in the coding sequence shows allele frequency difference between AAs and whites of approximately 15% (38).

^hProbe overlaps one SNP, but impact may be negligible due to location at end of probe, rarity of the minor allele, and/or no appreciable population difference (>10% in African vs. European) reported; therefore, probes were retained.

ⁱProbes reported to overlap a SNP or repeat (R) according to Byun et al. (19), or based on updated information in Ensembl and dbSNP that are likely to disrupt probe binding.

Supplementary Table S2, four additional probes that did not previously meet the threshold of $q < 0.05$ showed significant racial differences among cases with HR⁻ tumors, including TUSC3_E29_R, RAF1_P330_F, SMARCA3_P17_R, and IMPACT_P234_R. Notably, more CpG loci showed racial variation within HR⁻ tumors than HR⁺ tumors. Only HBII-52_E142_F showed significant differential methylation within HR⁺ tumors of AAs versus non-AAs. The box and whisker plots summarizing the distribution of methylation β values for probes significantly varying by race (at $q < 0.05$), overall or within HR-defined tumor subsets, are shown in Fig. 1A–C. Technical validation comparing methylation obtained from the GoldenGate array with Q-MSP confirmed the comparability of the methylation measurements obtained by these two methods (Supplementary Fig. S2).

Although there were too few genes differentially methylated by racial group to perform formal gene ontology or pathway analyses, several of the genes showing epigenetic racial differences have roles in DNA repair, transcription, or mediate other DNA interactions (*GSTM1*, *DNAJC15*, and *SMARCA3/HLTF*), are involved in cell adhesion (*DSC2* and *TES*), or are kinases important in signal transduction (*AXL* and *RAF1*).

Because AAs experience more adverse breast cancer outcomes than non-AAs, we determined whether the top candidate CpG markers might be differentially associated with disease-specific

survival among AAs versus non-AAs. The Kaplan–Meier plots in Supplementary Fig. S3 indicate that a low or intermediate (vs. high) level of methylation at several probes (*HBII-52_E142_F*, *TES_P182_F*, *DNAJC15_P65_F*, *DNAJC15_E26_R*, and *DSC2_E90_F*) was marginally associated with worse survival in AAs but not in non-AAs.

Validation in TCGA breast tumors

Breast tumor methylation and gene expression data from TCGA (21) were used to validate both our methylation findings and to test for correlations with expression for genes differentially methylated by race in the CBCS. Comparison of 450K methylation β values for probes that either directly matched or were located within 200 bp of GoldenGate probes in 42 AA and 291 white TCGA patients confirmed our findings of racial differences in methylation for *AXL*, *GSTM1*, *KCNK4*, and *DNAJC15* (Table 3 and Fig. 1D). Methylation was also significantly inversely correlated with gene expression for the majority of our candidate genes (Supplementary Table S3).

Racial differences in methylation in PBLs or matched tumor/PBL pairs

Methylation patterns are considered to be tissue-specific, yet, methylation at certain loci differs by race rather than tissue type,

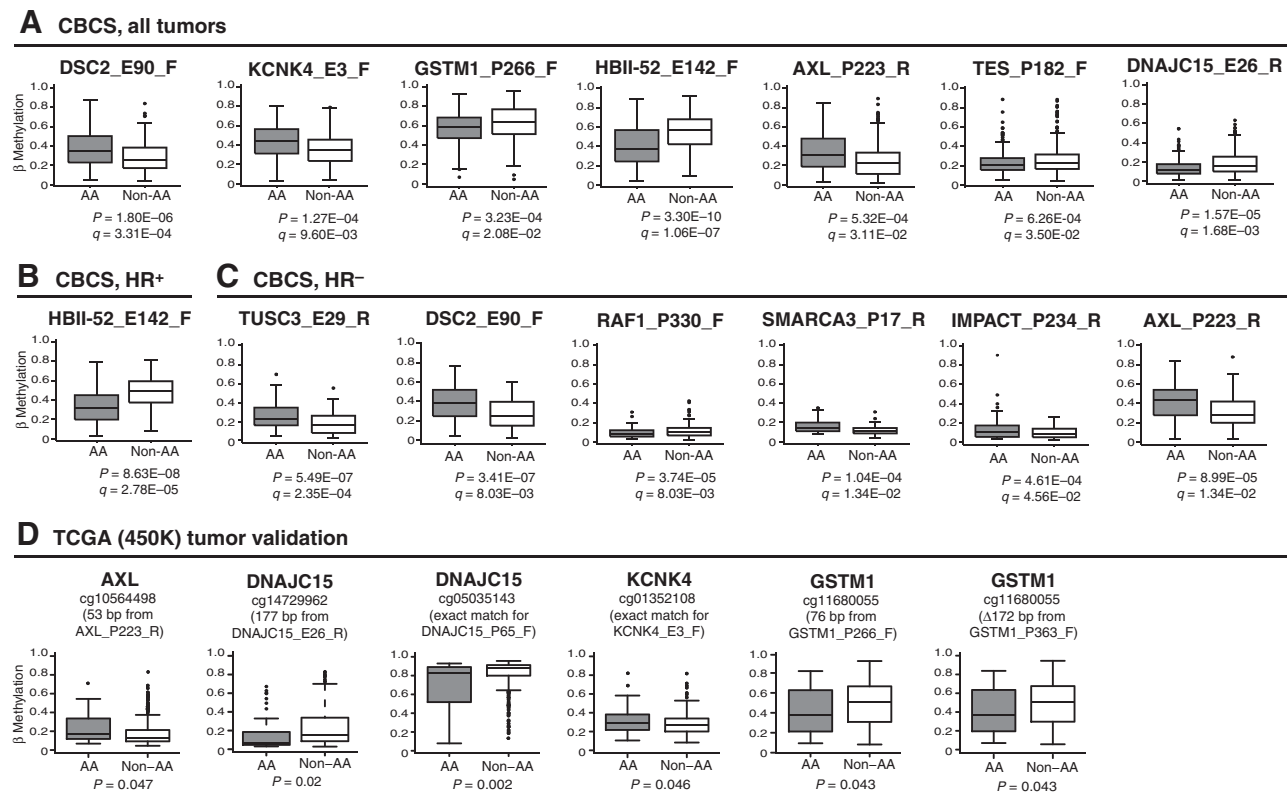


Figure 1. The box and whisker plots showing CpG probes exhibiting racial differences in breast tumor methylation. CpG probes shown differed significantly (at $q < 0.05$) in tumor methylation between AA and non-AA cases in GLM after controlling for age, menopausal status, stage, intrinsic subtype, and multiple comparisons (FDR). Each box plot shows the median β -value (dark bar within box), the interquartile range (outer boundaries of box), and the whiskers extend to the group minimum and maximum β values. Probes shown either do not overlap a sequence variant or overlap a single SNP that is likely to have negligible impact on probe performance. Probes show significant racial differences in methylation in all CBCS breast tumors ($n = 517$; A), HR⁺ breast tumors ($n = 337$; B), and HR⁻ breast tumors ($n = 163$; C). Shown are adjusted P values and q values from GLM. D, validation of racial variation in methylation for candidate genes in TCGA breast tumors. Data are shown for 450K CpG probes that exactly matched or were closest to (within 200 bp) probes on the GoldenGate array. P values were determined by the t test.

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Table 3. TCGA validation of differential breast tumor methylation in genes showing racial variation in the CBCS

450K probe	Chr	Gene	GoldenGate probe	Distance b/w GG and TCGA probes (bp) ^a	Mean β AA	Mean β non-AA	P^b
cg10564498	19	AXL	AXL_P223_R	53	0.223	0.182	0.05
cg03247049	19	AXL	AXL_E61_F	14	0.086	0.063	0.22
cg12722469	19	AXL	AXL_E61_F	169	0.189	0.147	0.12
cg05035143	13	DNAJC15	DNAJC15_P65_F	0	0.086	0.818	0.002
cg12504148 ^c	13	DNAJC15	DNAJC15_P65_F	34	0.610	0.690	0.02
cg14729962	13	DNAJC15	DNAJC15_E26_R	177	0.151	0.222	0.02
cg00566759	18	DSC2	DSC2_E90_F	58	0.065	0.055	0.34
cg13870990	18	DSC2	DSC2_E90_F	121	0.207	0.207	0.99
cg00196671	18	DSC2	DSC2_E90_F	-58	0.062	0.060	0.87
cg11680055	1	GSTM1	GSTM1_P266_F	76	0.410	0.490	0.04
cg24275769	18	IMPACT	IMPACT_P234_R	-2	0.137	0.108	0.18
cg13981356	18	IMPACT	IMPACT_P234_R	-78	0.205	0.196	0.69
cg03400437	18	IMPACT	IMPACT_P234_R	7	0.059	0.036	0.21
cg22757447	18	IMPACT	IMPACT_P234_R	-132	0.170	0.148	0.45
cg01352108	11	KCNK4	KCNK4_E3_F	0	0.329	0.281	0.05
cg09396196	11	KCNK4	KCNK4_E3_F	176	0.104	0.046	0.06
cg06129498	11	KCNK4	KCNK4_E3_F	59	0.169	0.142	0.09
cg14673256	11	KCNK4	KCNK4_E3_F	179	0.105	0.046	0.04
cg02830576	3	RAF1	RAF1_P330_F	0	0.255	0.246	0.40
cg18568714	3	RAF1	RAF1_P330_F	-2	0.072	0.061	0.27
cg13703021	3	RAF1	RAF1_P330_F	157	0.146	0.125	0.21
cg11297934	3	RAF1	RAF1_P330_F	-139	0.020	0.018	0.12
cg24506533	3	RAF1	RAF1_P330_F	-122	0.022	0.022	0.93
cg23032965	3	RAF1	RAF1_P330_F	-173	0.023	0.022	0.49
cg15438497	3	HMTF	SMARCA3_P17_R	46	0.103	0.081	0.20
cg24621354	7	TES	TES_P182_F	-17	0.077	0.094	0.15
cg20879085	7	TES	TES_P182_F	42	0.061	0.063	0.93
cg19743881	7	TES	TES_P182_F	39	0.092	0.094	0.92
cg16379337	7	TES	TES_P182_F	51	0.084	0.070	0.60
cg00254079	7	TES	TES_P182_F	-23	0.035	0.049	0.16
cg03127174	8	TUSC3	TUSC3_E29_R	-28	0.079	0.083	0.83
cg18145877	8	TUSC3	TUSC3_E29_R	-30	0.057	0.061	0.88
cg03032098	8	TUSC3	TUSC3_E29_R	140	0.112	0.094	0.46

^aInfinium 450K probes targeting CpG loci within 200 bp of the GoldenGate probes were assessed for racial differences in methylation in breast tumors from 42 AAs and 291 whites in TCGA; negative value indicates the 450K probe was 5' of the GoldenGate probe.

^b P values determined by the t test. There were no 450K probes for HBI-52 (alias SNORD115-1) within 200 bp of the GoldenGate probe.

^cThis 450K probe interrogates a CpG site (cg12504148) that is 55 bp upstream of the CpG targeted by the DNAJC15_E26_R GoldenGate probe.

we expect that these differences might also be detectable in other normal cells, even if derived from a different tissue. To determine whether the racial differences in CpG methylation were restricted to tumors or occurred more broadly, we compared methylation in PBLs from 29 AAs and 40 non-AA cases matched on age and menopausal status. As shown in Table 4, the majority of CpG markers that were differentially methylated by race in tumors also differed significantly in PBLs, and the directionality of the difference in PBLs reflected that of the tumors. For example, similar to the pattern in tumors, DSC2_E90_F, GSTM1_P266_F, KCNK4_E3_F, and AXL_P223_R all showed higher methylation in PBLs of AAs than in non-AAs even though the absolute levels of methylation varied somewhat between tumor and PBL. Differential methylation by race was also observed for CpG sites in *AXL*, *GSTM1*, *DSC2*, and *DNAJC15* in an independent series of lymphoblastoid cell lines from 80 female AAs compared with 49 female Caucasian Americans described by Heyn and colleagues (Supplementary Table S4; ref. 23).

We also compared methylation between matched pairs of breast tumors and PBLs within individual AA and non-AA cases ($n = 61$) for several probes exhibiting the largest methylation differences by race. Methylation in tumor/PBL pairs was significantly correlated (with $P < 0.05$) for the majority of probes (not shown). The box and whisker plots in Fig. 2A show that among the 61 cases for whom tumor and PBL samples are matched, the

methylation differences observed in tumors are also evident within PBLs. The bar chart in Fig. 2B provides a comparison of individual β values in matched tumors and PBLs from breast cancer cases, further highlighting the differential methylation detected between AAs and non-AAs.

Discussion

In this study, using a methylation array targeted to the promoters of genes important in cancer, we identified several genes differing in tumor DNA methylation between AAs and non-AAs even after controlling for potential confounders, including intrinsic subtype distributions that are known to vary by race (3). Previous studies, including the CBCS, have shown that breast tumor methylation varies between intrinsic subtypes (10, 18, 21, 26) or HR status (27), and by clinicopathologic characteristics such as tumor size and grade (18). Importantly, most of the CpG loci differing by race in CBCS breast tumors were also similarly differentially methylated in normal white blood cells of cases, although the absolute levels of methylation were not necessarily the same. A recent study that used the whole-genome Illumina 450K methylation array to assess racial differences in breast tumor methylation excluded probes that overlapped SNPs or that were otherwise ambiguous (15); however, we opted to retain such probes but to note their status. The impact of a probe

Table 4. Differential methylation in PBLs of AA versus non-AA cases in the CBCS

CpG probe	AA mean β (n = 29)	Non-AA mean β (n = 40)	Delta β	Unadjusted P value	Adjusted P value ^a	Overlapping variant
DSC2_E90_F	0.4721	0.2904	0.1817	<0.0001	<0.0001	None
KCNK4_E3_F	0.5203	0.4144	0.1059	<0.0001	<0.0001	None
GSTM1_P266_F	0.3252	0.4637	-0.1385	0.004	0.01	None
TUSC3_E29_R	0.1552	0.1040	0.0512	0.001	0.001	None
SMARCA3_P17_R	0.1079	0.0898	0.0181	0.003	0.002	None
IMPACT_P234_R	0.0972	0.0727	0.0245	0.001	0.001	None
RAF1_P330_F	0.0901	0.1105	-0.0204	0.43	0.19	None
HBII-52_E142_F	0.5930	0.7702	-0.1772	0.0001	<0.0001	None
AXL_P223_R	0.6699	0.5306	0.1393	<0.0001	<0.0001	SNP ^b
DNAJC15_E26_R	0.1308	0.1582	-0.0274	0.0003	<0.0001	SNP ^b
DNAJC15_P65_F	0.9427	0.9565	-0.0138	0.0003	<0.0001	SNP
TES_P182_F	0.1642	0.1439	0.0203	0.10	0.05	SNP ^b

Comparison of transformed β values in AAs versus non-AAs was performed using the *t* test.

^aAdjusted for age and menopausal status.

^bProbe overlaps one SNP, but impact may be negligible due to SNP location at end of probe, rarity of the minor allele, and/or no appreciable difference in population (African vs. European) frequencies reported.

that overlaps a target sequence potentially containing a SNP is difficult to predict, as it would depend on the SNP frequency in a given population as well as the probe sequence and precise location of the underlying SNP. Because many probes on the Illumina methylation arrays often overlap multiple CpG sites, the effect of an additional SNP-based single-nucleotide mismatch on probe binding and methylation detection may be minimal. Consistent with this, we have previously observed in the CBCS that some probes overlapping a SNP performed similarly in their detection of methylation (i.e., generated similar β values) to nearby probes in the same gene that do not (22). Genotyping for SNPs located within gene promoters was not performed in the CBCS and thus we could not rule out that racial variation in methylation might be related to the effects of underlying SNPs for certain probes; however, for most probes that overlapped a SNP, the variant alleles were relatively rare (minor allele frequency <10%) but in some cases differed by ancestry. Thus, to focus on CpG probes that would most reliably detect methylation differences (rather than underlying SNPs), we reviewed all probe target sequences against NCBI databases, assessed reported racial differences in allelic frequencies if available, and then removed those for which the underlying feature was likely to impede a reliable measurement of methylation. In the end, a total of 11 CpG probes were retained that were likely to provide a reasonably accurate assessment of racial variation in methylation.

Several prior studies using targeted methylation approaches to examine a small number of candidate genes known to be involved in breast cancer reported differences in breast tumor methylation between AAs and those of European ancestry. Consistent with our findings of more methylation differences by race within HR⁻ tumors, Mehrotra and colleagues (14) found significantly higher methylation in AAs than in Caucasian women for four genes within a five-gene marker panel (*HIN-1*, *TWIST1*, *CCND2*, *RASSF1A*, *RARB*); these differences were only evident within ER⁻ tumors and among women diagnosed before age 50 years. Similarly, Wang and colleagues (13) examined an eight-marker panel (p16, *RASSF1A*, *RARB2*, *ESR1*, *CDH13*, *HIN1*, *SFRP1*, and *LINE1*) and reported differential methylation for *CDH13* between ER⁻ tumors from younger AAs and European-American (EA) women. Using the Illumina 450K whole-genome methylation array, the same array platform used in TCGA, Ambrosone and colleagues (15) also reported that many more loci were differentially methylated in AAs compared with EAs in ER⁻ than in ER⁺

breast cancers. The genes exhibiting differential promoter methylation by race in the CBCS are unique, and not among the candidate markers examined previously or the top loci reported from the 450K analysis in the Ambrosone study (15). Importantly, it should be noted that our analyses controlled for differences in intrinsic subtype, stage, and age, whereas these prior studies did not. Therefore, it is unclear whether some loci reported previously as varying by race or ancestry may actually reflect differences in tumor intrinsic subtype distributions between the racial groups, such as within ER⁻ tumors that are comprised the basal-like, claudin-low, and HER2⁺/ER⁻ subtypes. Intriguingly, several genes showing racial variation in tumor methylation (*HBII-52*, *TES*, *DNAJC15*, and *DSC2*) may be associated with differences in survival among AAs, while no such differences were observed among non-AAs. Further work is needed to confirm these results and to assess the possibility that underlying copy number or other changes contributed to these findings.

In the CBCS, we also observed racial differences in methylation in normal PBL samples from cases, with the overall patterns of tumor and PBL methylation for these probes being correlated, although the absolute levels of methylation in tumor versus PBL differed for some loci. Differences in the quality of DNA derived from FFPE tumors versus PBLs could potentially contribute to such methylation differences; however, PBL samples did not consistently show higher or lower methylation levels than in tumors. Although variation in PBL methylation profiles primarily reflect the admixture of white blood cell populations (28, 29), we still observed epigenetic differences by race. The finding of racial variation in methylation in both tumor and PBLs suggests underlying ancestral differences in the epigenetic state of some genes, with about half of differentially methylated loci being potentially explained by known sequence variations that could disrupt probe binding and methylation measurements. This is consistent with several prior studies noting that such genetic variants contributed to a substantial portion of racial differences in DNA methylation (23, 30–32). Racial differences in DNA methylation patterns in normal tissues have also been detected in PBLs from women in a multiethnic New York City Birth Cohort (33), in umbilical cord blood from newborns (34), and in breast tissue from reduction mammoplasty (11), raising the question of whether such normal epigenetic variation contributes to cancer risk. In a study of normal human prostate tissue and prostate cancer, several genes exhibited differential methylation between AAs and EAs (12).

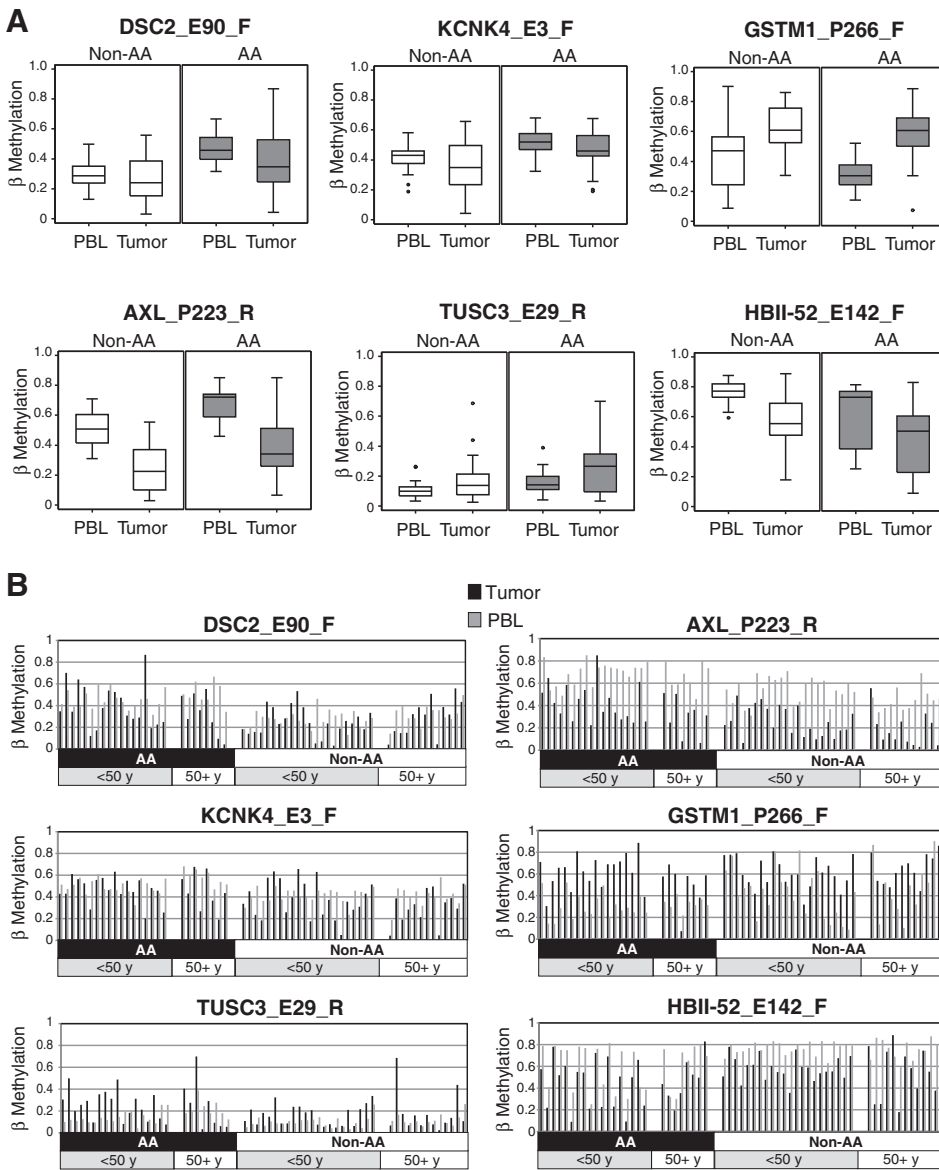


Figure 2. Methylation in matched pairs of breast tumors and PBLs from AA and non-AA cases in the CBCS. A, the box and whisker plots showing the distribution of methylation β values in 61 matched pairs of breast tumors and PBLs from AA and non-AA cases. B, bar charts showing individual methylation β values of matched tumor (black) and PBL (gray) pairs by race (26 AA, 35 non-AA) and age group (40, <50 years; 21, 50+ years).

The genes displaying racial variation in tumor methylation in the CBCS included several involved in transcription or interactions with DNA (*DNAJC15*, *SMARCA3*, or *HLTF*), signal transduction (*AXL* and *RAF1*), carcinogen detoxification (*GSTM1*), cell adhesion (*DSC2*), or are known tumor suppressors (*TUSC3* and *TES*). Desmocollin 2 (*DSC2*), a membrane glycoprotein involved in cell-cell adhesion and maintenance of normal epithelial architecture is an independent prognostic marker for esophageal (35) and pancreatic cancers (36), and high *DSC2* expression may be a marker of basal-like breast tumors (37). The carcinogen detoxifying enzyme, *GSTM1*, exhibits an intragenic deletion variant resulting in loss of enzyme activity (25), elevated cancer risk, and shows racial variation between blacks and whites (38); however, whether the variant is linked with promoter methylation state is unclear. Testin (*TES*) and *TUSC3* are tumor-suppressor genes that are silenced, in part, via promoter methylation (39); loss of expression of *TES* is an independent poor prognostic marker in

breast cancer (40), while silencing of *TUSC3* may be a poor prognostic factor in ovarian cancer (41). *AXL* is a receptor tyrosine kinase and an epithelial-to-mesenchymal transition (EMT)-induced regulator of breast cancer metastasis and patient survival (42–45). *AXL* expression is regulated, in part, by Sp1 transcription factor binding and methylation (42, 44, 46). In contrast to most other genes showing epigenetic racial variation in the CBCS, *AXL* methylation was not inversely correlated with mRNA expression in the TCGA tumor set, most likely because the 450K *AXL* CpG probes analyzed in relation to gene expression appear to be outside of these critical Sp1 binding sites. Intriguingly, *AXL* was reported to be aberrantly hypermethylated in association with prenatal tobacco smoke exposure (47), suggesting that epigenetic racial variation may be associated with lifestyle or environmental exposures and could potentially contribute to cancer risk.

The strengths of this study include the large and well-characterized population-based series of mostly early-stage breast cancer

cases, providing the power to detect even modest differences in methylation. Epigenetic racial differences for several of the genes in the CBCS were independently confirmed in TCGA breast tumors although the number of AAs in this dataset was limited. Moreover, the availability of demographic, clinical and subtype information allowed us to control for factors that are known to vary by patient or tumor subsets. However, our study was limited by use of the targeted cancer-focused array rather than a whole-genome methylation array.

The implications of the racial differences in methylation discovered in this study for breast cancer development, progression, or outcomes in AA or non-AA women are unclear, and further work is needed to confirm these findings. It is of interest that the epigenetic differences were most evident within HR⁻ tumors despite controlling for intrinsic subtype; however, it is possible that these racial variations signify different proportions of HR⁻ subtypes in AA and non-AA women. Interestingly, most of the genes exhibiting differential tumor methylation by race in the CBCS (*DSC2*, *AXL*, *DNAJC15*, *TES*, and *TUSC3*) are among the genes defining subclasses of triple-negative breast cancer as reported by Lehmann and colleagues (48). Whether the prevalence of triple-negative subtypes, which were not defined within the CBCS, vary between AAs and non-AAs is presently unknown. The results of this study highlight the possibility that methylation patterns of breast tumors, particularly the more aggressive HR⁻ tumors, may differ by ancestry, and that the racial differences may not be a tumor-specific phenomenon, suggesting that such variations could also contribute to cancer risk. Clearly, more research is needed to validate these findings and determine whether they contribute to the known racial disparity in breast cancer survival.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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In Memoriam

This work is dedicated to the memory of our colleague, the late Dr. Robert Millikan, principal investigator of the CBCS.

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Acknowledgments

The authors thank Dr. Andy Olshan for critical review of this article and the participants and study staff of the CBCS for their continuing support of the study.

Grant Support

This research was supported by grants to K. Conway from Susan G. Komen Foundation (grant #KG081397) and from the University Cancer Research Fund of North Carolina. The CBCS was also funded by the University Cancer Research Fund of North Carolina and the National Cancer Institute Specialized Program of Research Excellence (SPORE) in Breast Cancer (NIH/NCI P50-CA58223).

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Received October 31, 2014; revised February 11, 2015; accepted March 3, 2015; published OnlineFirst March 25, 2015.

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