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Detection of promoter methylation of tumor suppressor genes in serum DNA of breast cancer cases and benign breast disease controls

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Tumors are capable of shedding DNA into the blood stream. This shed DNA may be recovered from serum or plasma. The objective of this study was to evaluate whether pyrosequencing promoter DNA in a panel of 12 breast cancer-related genes (*APC*, *BRCA1*, *CCND2*, *CDH1*, *ESR1*, *GSTP1*, *HIN1*, *P16*, *RAR*β, *RASSF1*, *SFRP1* and *TWIST*) to measure the degree of methylation would lead to a useful serum-based marker of breast cancer. Serum was obtained from women who were about to undergo a breast biopsy or mastectomy at three hospitals from 1977 to 1987 in Grand Rapids, MI USA. We compared the methylation status of 12 genes in serum DNA obtained from three groups of postmenopausal women (mean age at blood collection: 63.0 y; SD 9.9; range 35–91): breast cancer cases with lymph node-positive disease (n = 241); breast cancer cases with lymph node-negative disease (n = 63); and benign breast disease control subjects (n = 234). Overall, median levels of promoter methylation were low, typically below 5%, for all genes in all study groups. For all genes, median levels of methylation were higher (by 3.3% to 47.6%) in lymph node-positive breast cancer cases than in the controls. Comparing mean methylation level between lymph-node positive cases and controls, the most statistically significant findings, after adjustment of the false-positive rate (q-value), were for *TWIST* (p = 0.04), *SFRP1* (p = 0.16), *ESR1* (p = 0.17), *P16* (p = 0.19) and *APC* (p = 0.19). For two of these four genes (*TWIST*, *P16*), the median methylation level was also highest in lymph-node positive cases, intermediate in lymph node-negative cases and lowest in the controls. The percent of study subjects with mean methylation scores ≥ 5% was higher among lymph node-positive cases than controls for ten genes, and significantly higher for *HIN1* and *TWIST* (22.0 vs. 12.2%, p = 0.04 and 37.9 vs. 24.5%, p = 0.004, respectively). Despite relatively consistent variation in methylation patterns among groups, these modest differences did not provide sufficient ability to distinguish between cases and controls in a clinical setting.

Introduction

Aberrant DNA methylation is a common feature of malignant cells and, thus, the methylation status of key cell cycle regulation genes is often proposed as a reliable and sensitive early marker for breast cancer.[1,2] Classic tumor suppressor genes and other regulatory genes are frequently silenced by methylation at their promoter regions in cancerous breast tissue.[3,4]

Promising estimates for sensitivity and specificity for the detection of cancerous breast tissue have been observed using promoter methylation of a small panel of common cancer-related genes to discriminate between normal and diseased tissue. For example, Fackler and colleagues[5] reported a sensitivity for detection of breast cancer of 85% and a specificity of 89% based on a panel of four genes (*CCND2*, *HIN1*, *RASSF1* and *TWIST*) in 18 normal mammoplasty and 21 tumor specimens. Similarly, Esteller and colleagues[6] reported a sensitivity of 73% using a different panel of four genes (*GSTP1*, *BRCA1*, *CDH1* and *P16*) based on 45 breast cancer tumors. In another report by Fackler[7] with five candidate genes evaluated, one or more methylated genes were detected in 100% of invasive lobular cancer and 95% of lobular in situ tumors (total of 103 cases). Collectively, these data indicate that the promoter methylation status of even a relatively small number of genes is sufficient to distinguish breast cancer tissue from normal tissue.

Other data indicate that higher levels of DNA, as much as six times higher on average, can be detected in the blood of breast cancer cases than in the blood of healthy subjects.[8] Furthermore, DNA from plasma or serum from breast cancer patients has been suggested to be of tumor origin because in several small studies the promoter methylation patterns in serum or plasma DNA have shown high concordance to those found in corresponding tumors.[9] However, the proportion of free circulating DNA that is from the tumor has been reported to vary widely, from less than 0.5% to 90%.[10,11] This variability and other technical issues have led some researchers to question the feasibility of developing a circulating DNA marker for early breast cancer.[12,13] A number of small studies

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Table 1. Selected characteristics of the study population

Characteristic	BBD controls (n = 234)	BC cases negative nodes (n = 63)	BC cases positive nodes[d] (n = 241)
Age at blood collection; mean (S.D.) in years	59.3 (9.1)	67.0 (8.4)	65.4 (10.1)
Age at menarche; mean (S.D.) in years[a]	13.0 (1.7)	13.3 (1.7)	13.1 (1.4)
Age at menopause; mean (S.D.) in years	45.7 (6.9)	46.6 (6.5)	47.3 (5.6)
Body mass index (kg/m^2)[b]	24.9 (3.7)	25.6 (4.2)	25.7 (4.6)
Nulliparous (%)	15.8%	25.4%	18.3%
No. of full-term pregnancies	3.2 (1.6)	2.9 (1.8)	3.2 (1.8)
Age at first full-term pregnancy; mean (S.D.) in years	23.2 (4.5)	25.0 (5.3)	24.2 (4.8)
Family history of breast cancer (%)[c]	24.0%	33.9%	26.7%
Ever smoked (%)	33.0%	19.1%	41.0%

[a]Unknown for 5 controls, 0 node-negative, and 2 node-positive cases; [b]unknown for 1 control, 0 node-negative and 1 node-positive cases; [c]unknown for 17 controls, 4 node-negative and 9 node-positive cases; [d]includes 82 cases with uncertain node status.

have directly assessed the ability of promoter methylation status, typically in one to four genes, in serum or plasma DNA to discriminate between breast cancer cases and controls.[14] For example, Hoque and colleagues[15] reported a sensitivity of 62% and specificity of 85% with four candidate genes (*APC*, *GSTP1*, *RASSF1A* and *RARβ2*) using quantitative methylation-specific PCR (QMSP) in a study of 47 breast cancer cases and 38 control subjects. By contrast, a recently published larger study by Brooks and colleagues[16] was not successful in distinguishing between 50 breast cancer cases and 300 cancer-free controls based on the methylation status via QMSP of same four genes that were studied by Hoque and colleagues.[15] Based on a re-analysis of a subset of specimens using quantitative pyrosequencing, Brooks and colleagues[16] attributed their inability to detect differences in promoter methylation status between cases and controls to a high prevalence of false-positives using QMSP methods on serum DNA. In contrast to QMSP, pyrosequencing allows for the interrogation of the methylation status of multiple CpG sites, and has an internal control to test for complete bisulfite treatment.

Thus, the objective of the present study was to evaluate whether an expanded panel of 12 tumor suppressor genes (*APC*, *BRCA1*, *CDH1*, *CCND2*, *ESR1*, *GSTP1*, *HIN1*, *p16*, *RASSF1*, *RARβ*, *SFRP1* and *TWIST1*) frequently found to be methylated in breast cancers but not in normal breast tissue, when combined with the quantitative pyrosequencing methods to assess promoter methylation status, would lead to a sufficiently sensitive and specific serum-based methylation marker. We analyzed promoter methylation in DNA from serum collected at the time of diagnosis from 325 breast cancer cases and 249 benign breast disease controls who donated blood from 1977 to 1987 to the Mayo Serum Bank, a resource established to identify new early markers of breast cancer. We examined promoter methylation in DNA from serum from lymph-node positive and lymph-node negative breast cancer cases separately based on reports that the methylation pattern of tumor suppressor genes may be associated with the clinical characteristics of the tumor.[17]

Results

Characteristics of study participants. This study compared the promoter methylation status of 12 genes in serum DNA obtained from postmenopausal women in three study groups: breast cancer cases with lymph node-positive disease (n = 241), breast cancer cases with lymph node-negative disease (n = 63) and benign breast disease control subjects (n = 234). A description of selected baseline characteristics for women in each of the study groups is presented in **Table 1**. Overall, the mean age at blood collection was 63.0 y: SD 9.9; range 35–91. On average, lymph node-positive and negative breast cancer cases were six to seven years older than the benign breast disease controls. Lymph-node positive breast cancer cases also tended to be slightly more likely to be nulliparous (18.3 vs 15.8%), to have a later age at first birth (24.2 vs 23.2 y), to have a later age at menopause (47.3 vs 45.7 y) and to have a family history of breast cancer (26.7% vs 24.0%) than benign breast disease controls. Compared with benign breast disease control subjects, lymph node-negative breast cancer cases were also more likely to be nulliparous (24.4% vs. 15.8%), to have a later age at first birth (25.0 vs. 23.2 y), to have a family history of breast cancer (33.9% vs. 24.0%), and to have a later age at menopause (46.6 vs. 45.7 y). However, lymph node-positive breast cancer cases were more likely to have ever smoked than benign breast disease controls (41.0% vs. 33.0%) whereas lymph node-negative cases were less likely to have ever smoked (19.1% vs. 33.0%). The three study groups were very similar in other factors, including age at menarche, body mass index, and number of full-term pregnancies.

Comparison of median levels of gene promoter methylation across the three groups. Pyrosequencing analysis was used to assess the percent of DNA that was methylated at specific CpG sites in the promoter regions of 12 genes. The average of the percent methylation over all the promoter CpG sites for each gene (ranging from 3 to 27 CpG sites per gene) was calculated. Median levels of the average DNA methylation for 12 genes for each of the three study groups and the corresponding 10th to 90th percentiles are presented in **Table 2**. Overall, median levels of promoter methylation were very low, typically below 5%, for each gene in all of the study groups. For all of the 12 genes, as hypothesized, median levels of methylation were slightly higher in breast cancer cases with positive lymph nodes than in benign breast disease control subjects (by 3.3% for RASSF1 to 47.7% for

Table 2. Median methylation levels (average over all CpG sites per gene) by case-control status

[# CpG sites]	BBD controls (n = 234)		BC cases negative nodes (n = 63)		BC cases positive nodes[a] (n = 241)	
	n	Median (10th to 90th)	n	Median (10th to 90th)	n	Median (10th to 90th)
APC [10]	198	1.7 (0.1–2.1)	56	1.3 (0.3–1.8)	190	1.8 (0.3–2.6)
BRCA1 [15]	147	3.9 (0.8–8.6)	34	6.4 (1.1–14.4)	158	4.4 (0.9–10.3)
CCND2 [6]	171	2.1 (0.5–3.7)	51	2.4 (0.9–3.5)	178	3.2 (0.8–5.5)
CDH1 [7]	191	3.6 (0.5–7.1)	51	4.8 (0.8–10.4)	200	3.9 (0.4–7.9)
ESR1 [5]	212	5.3 (0.8–11.2)	59	6.2 (1.3–19.4)	200	6.2 (1.2–11.2)
GSTP1 [4]	186	2.1 (0.6–2.8)	55	1.9 (0.6–3.4)	178	2.7 (0.8–4.7)
HIN1 [27]	131	2.4 (0.5–6.1)	36	3.3 (0.5–7.6)	141	3.8 (0.5–8.5)
P16 [14]	189	4.0 (0.3–8.7)	53	4.1 (0.3–7.6)	193	4.7 (0.4–8.3)
RARβ [3]	222	1.5 (0.0–2.6)	60	1.5 (0.0–2.4)	223	1.7 (0.0–2.4)
RASSF1 [9]	200	3.0 (0.5–4.9)	50	3.0 (0.8–6.4)	192	3.1 (0.6–5.4)
SFRP1 [8]	184	4.2 (0.4–13.7)	53	4.1 (0.4–9.8)	197	6.2 (0.5–18.6)
TWIST [22]	200	3.9 (1.6–6.8)	56	4.4 (2.4–7.5)	203	4.9 (1.8–8.7)

[a]Includes 82 cases with uncertain node status.

SFRP1). In addition, in seven of the 12 genes (*BRAC1*, *CCND2*, *CDH1*, *ESR1*, *HIN1*, *P16* and *TWIST*), the median methylation level was also higher in breast cancer cases with negative lymph nodes than in benign breast disease subjects. In four genes (*CCND2*, *HIN1*, *P16* and *TWIST*), median levels of methylation were highest in lymph-node positive breast cancer cases, intermediate in the lymph-node negative breast cancer cases and lowest in benign breast disease control subjects. Median levels of the average DNA methylation for 12 genes were also examined by two categories of number of positive nodes (1–2 vs 3+) among the 159 node-positive cases only. In eight of the 12 genes, the median methylation levels were slightly higher in cases with 3+ positive nodes than in cases with 1 or 2 positive nodes, although this difference was statistically significant only for *ESR1* (2.7% vs 2.0%).

Comparison of quartile levels of gene promoter methylation between lymph node-positive breast cancer cases and benign breast disease controls. The distribution of lymph node-positive breast cancer cases in each quartile of gene methylation (quartile cut points were based on the distribution of gene methylation in benign breast disease controls) is presented in **Table 3**. The six most statistically significant findings, after adjustment of the false-positive rate (q-value), were for *TWIST* (p = 0.04, q = 0.43), *SFRP1* (p = 0.16, q = 0.43), *ESR1* (p = 0.17, q = 0.43), *P16* (p = 0.19, q = 0.43), *APC* (p = 0.19, q = 0.43) and *HIN1* (p = 0.21, q = 0.43). Odds ratios and 95% confidence intervals comparing the three highest quartiles to lowest quartile of gene methylation for lymph node-positive breast cancer cases compared with benign breast disease controls are also shown. For *APC* and *TWIST*, the odds ratios comparing the highest to lowest quartiles were elevated and marginally statistically significant. Specifically, the OR comparing the highest to lowest quartile of gene methylation for *TWIST* was 1.75 (95% CI = 1.0–3.0). The comparable figure for *APC* was 1.78 (95% CI = 1.0–3.0). For *ESR1* and *SFRP1*, the odds ratios in the top three quartiles compared with the lowest quartile all tended to be elevated; only the top quartile for *ESR* was marginally statistically significant (RR = 1.77; 95% CI = 1.0–3.1). For two of the remaining genes (*BRCA1* and *CCND2)*, the highest odds ratio was also observed in the top quartile.

Results were generally similar when we examined each gene separately among women with 1–2 and 3+ positive nodes compared with benign breast disease controls. For example, the three most statistically significant genes after adjustment for the Q-value were *TWIST*, *SFRP1* and *ESR1* for women with 1–2

Table 3. Association between quartile levels of mean methylation scores and node-positive breast cancer

	BBD controls N %	BC cases positive nodes[b] N %	BC cases positive nodes[b] OR (95% CI)	p (q)
APC (%)				0.19 (0.43)
Q1 ≤ 0.4	51 (25.8)	38 (20.0)	1.0[a]	
Q2 (0.4–0.7)	49 (24.7)	41 (21.6)	1.12 (0.6–2.0)	
Q3 (0.7–1.0)	49 (24.7)	46 (24.2)	1.26 (0.7–2.3)	
Q4 (1.0–39.6)	49 (24.7)	65 (34.2)	1.78 (1.0–3.1)	
BRCA1 (%)				0.44 (0.75)
Q1 ≤ 1.2	37 (25.2)	34 (21.5)	1.0[a]	
Q2 (1.3–3.2)	37 (25.2)	34 (21.5)	1.00 (0.5–1.9)	
Q3 (3.2–4.1)	36 (24.5)	37 (23.4)	1.12 (0.6–2.2)	
Q4 (4.1–35.6)	37 (25.2)	53 (33.5)	1.56 (0.8–2.9)	
CCND2 (%)				0.56 (0.84)
Q1 ≤ 0.9	43 (25.1)	38 (21.3)	1.0[a]	
Q2 (0.9–1.5)	44 (25.7)	48 (27.0)	1.23 (0.7–2.2)	
Q3 (1.5–2.4)	42 (24.6)	38 (21.3)	1.02 (0.6–1.9)	
Q4 (2.4 -32.9)	42 (24.6)	54 (30.3)	1.46 (0.8–2.6)	
CDH1 (%)				0.83 (0.94)
Q1 ≤ 1.0	48 (25.1)	44 (22.0)	1.0[a]	
Q2 (1.0–2.3)	48 (25.1)	57 (28.5)	1.30 (0.7–2.3)	
Q3 (2.3–4.6)	47 (24.6)	47 (23.5)	1.09 (0.6–1.9)	
Q4 (4.6–46.9)	48 (25.1)	52 (26.0)	1.18 (0.7–2.1)	
ESR1 (%)				0.17 (0.43)
Q1 ≤ 1.3	53 (25.0)	35 (17.5)	1.0[a]	
Q2 (1.3–2.1)	53 (25.0)	58 (29.0)	1.66 (0.9–2.9)	
Q3 (2.1–3.7)	53 (25.0)	45 (22.5)	1.29 (0.7–2.3)	
Q4 (3.7–95.4)	53 (25.0)	62 (31.0)	1.77 (1.0–3.1)	
GSTP1 (%)				0.86 (0.94)
Q1 ≤ 0.9	47 (25.3)	41 (23.0)	1.0[a]	
Q2 (0.9–1.3)	48 (25.8)	42 (23.6)	1.00 (0.6–1.8)	
Q3 (1.3–1.8)	44 (23.7)	47 (26.4)	1.23 (0.7–2.2)	
Q4 (1.8–50.0)	47 (25.3)	48 (27.0)	1.17 (0.7–2.1)	
HIN1 (%)				0.21 (0.43)
Q1 ≤ 0.7	33 (25.2)	36 (25.5)	1.0[a]	
Q2 (0.7–1.0)	33 (25.2)	31 (22.0)	0.86 (0.4–1.7)	
Q3 (1.0–2.0)	32 (24.4)	24 (17.0)	1.70 (0.3–1.4)	
Q4 (2.0–24.2)	33 (25.2)	50 (35.5)	1.39 (0.7–2.6)	
P16 (%)				0.19 (0.43)
Q1 ≤ 0.6	48 (25.4)	48 (24.9)	1.0[a]	
Q2 (0.6–1.0)	47 (24.9)	33 (17.1)	0.70 (0.4–1.3)	
Q3 (1.0–2.5)	47 (24.9)	63 (32.6)	1.34 (0.8–2.3)	
Q4 (2.5–39.9)	47 (24.9)	49 (25.4)	1.04 (0.6–1.8)	
RARβ (%)				0.94 (0.94)
Q1 ≤ 0.8	58 (26.1)	61 (27.4)	1.0[a]	
Q2 (0.8–1.2)	60 (27.0)	60 (26.9)	0.95 (0.6–1.6)	
Q3 (1.2–1.7)	48 (21.6)	43 (19.3)	0.85 (0.5–1.6)	
Q4 (1.7–14.2)	56 (25.2)	59 (26.5)	1.00 (0.6–1.7)	

[a]Reference category; [b]includes 82 cases with uncertain node status.

Table 3. Association between quartile levels of mean methylation scores and node-positive breast cancer (continued)

	BBD controls N %	BC cases positive nodes[b] N %	BC cases positive nodes[b] OR (95% CI)	p (q)
RASSF1 (%)				0.73 (0.94)
Q1 ≤ 1.1	51 (25.5)	42 (21.9)	1.0[a]	
Q2 (1.1–1.7)	49 (24.5)	49 (25.5)	1.21 (0.7–2.2)	
Q3 (1.7–2.9)	50 (25.0)	56 (29.2)	1.02 (0.8–2.4)	
Q4 (2.9–68.2)	50 (25.0)	45 (23.4)	1.09 (0.6–1.9)	
SFRP1 (%)				
Q1 ≤ 0.6	46 (25.0)	35 (17.8)	1.0[a]	0.16 (0.43)
Q2 (0.6–1.1)	46 (25.0)	42 (21.3)	1.20 (0.7–2.2)	
Q3 (1.1–4.5)	46 (25.0)	64 (32.5)	1.83 (1.0–3.3)	
Q4 (4.5–64.5)	46 (25.0)	56 (28.4)	1.60 (0.9–2.9)	
TWIST (%)				0.04 (0.43)
Q1 ≤ 2.3	50 (25.0)	44 (21.7)	1.0[a]	
Q2 (2.3–3.5)	51 (25.5)	45 (22.2)	1.0 (0.6–1.8)	
Q3 (3.5–4.9)	49 (24.5)	37 (18.2)	0.86 (0.5–1.5)	
Q4 (4.9 -15.4)	50 (25.0)	77 (37.9)	1.75 (1.0–3.0)	

[a]Reference category; [b]includes 82 cases with uncertain node status.

positive nodes and *ESR1*, *APC* and *TWIST* for women with 3 or more positive nodes. Finally, risk estimates presented in **Table 4** for node-positive breast cancer cases did not change meaningfully after adjustment for age at blood collection.

Comparison of quartile levels of gene methylation between lymph-node negative breast cancer cases and benign breast disease controls. The distribution of lymph-node negative breast cancer cases in each quartile of gene methylation (quartile cut-points were based on the distribution of gene methylation in benign breast disease controls) is presented in **Table 4**. Odds ratios and 95% confidence intervals comparing the three highest quartiles to lowest quartile of gene methylation for lymph-node negative breast cancer cases compared with benign breast disease controls are also shown. The two most statistically significant findings, after adjustment of the false-positive rate (q-value), were for *TWIST* (p = 0.04) and *HIN1* (p = 0.003). Finally, risk estimates presented in **Table 4** for node-negative breast cancer cases did not change meaningfully after adjustment for age at blood collection

Comparison of gene methylation levels of 5% or higher across study groups. Table 5 presents the distribution of women in each of the three study groups with a methylation level of at least five percent for specific genes. The proportion of individuals with ≥ 5% methylation was higher in lymph-node positive breast cancer cases than in benign breast disease control subjects for ten of 12 genes. However, for only two genes, *HIN1* and *TWIST*, was the proportion of subjects with methylation scores of 5% or greater statistically significantly higher in the node-positive breast cancer cases as compared with controls (22.0% vs 12.2%, p = 0.04; 37.9% vs 24.5%, p = 0.004, respectively). For all but four genes (*APC*, *GSTP1 P16* and *SFRP1*), the proportion of individuals with mean methylation scores of ≥ 5% was higher in lymph-node-negative breast cancer cases than in benign breast disease control subjects but there were no statistically significant differences.

We also considered the extent to which the methylation pattern in a combination of genes could distinguish between breast cancer cases from control subjects. The Random Forests statistical algorithm was applied to assess the discriminating ability of the multiple gene panel of DNA methylation profiles in distinguishing the node-positive cases from the controls.[18] Genes with greater than 25% of subjects missing methylation values (*CCND2*, *HIN1* and *BRAC1*) were excluded from the analysis. Subjects missing any values for the remaining nine genes were excluded—this resulted in the inclusion of 103 controls and 97 node-positive cases in the analysis. The out of bag error rate of the Random Forests classifier in predicting case/control status was 0.45, with a corresponding area under the curve (AUC) estimate of 0.45. A permutation test of the statistical significance of this classifier when compared with random chance resulted in a p value of 0.15. These results from the Random Forests classifier trained on methylation profiles corresponding to the panel of nine genes indicate no significant improvement in predictive power when compared with random chance.

Discussion

We conducted a case-control study to examine the extent to which promoter methylation of 12 breast cancer-related genes could serve as a marker for the early detection of breast cancer. To our knowledge, this is the first such study to use pyrosequencing to detect DNA methylation of cancer-related genes in serum. Serum was obtained from women who donated blood to the Mayo Serum Bank during 1977 to 1987 prior to undergoing a breast biopsy or mastectomy. We selected a panel of 12 tumor

Table 4. Association between quartile levels of mean methylation scores and node-negative breast cancer

	BBD controls N %	BC cases negative nodes N %	BC cases negative nodes OR (95% CI)	p (q)
APC (%)				0.65 (0.45)
Q1 ≤ 0.4	51 (25.8)	16 (28.6)	1.0[a]	
Q2 (0.4–0.7)	49 (24.7)	10 (17.9)	0.65 (0.3–1.6)	
Q3 (0.7–1.0)	49 (24.7)	17 (30.4)	1.11 (0.7–2.3)	
Q4 (1.0–39.6)	49 (24.7)	13 (23.2)	1.78 (1.0–3.1)	
BRCA1 (%)				0.86 (0.49)
Q1 ≤ 1.2	37 (25.2)	8 (23.5)	1.0[a]	
Q2 (1.3–3.2)	37 (25.2)	8 (23.5)	1.00 (0.3–2.9)	
Q3 (3.2–4.1)	36 (24.5)	7 (20.6)	0.90 (0.3–2.7)	
Q4 (4.1–35.6)	37 (25.2)	11 (32.4)	1.38 (0.5–3.8)	
CCND2 (%)				0.50 (0.43)
Q1 ≤ 0.9	43 (25.1)	8 (15.7)	1.0[a]	
Q2 (0.9–1.5)	44 (25.7)	13 (25.5)	1.59 (0.6–4.2)	
Q3 (1.5–2.4)	42 (24.6)	15 (29.4)	1.92 (0.6–1.9)	
Q4 (2.4 -32.9)	42 (24.6)	15 (29.4)	1.46 (0.8–2.6)	
CDH1 (%)				0.10 (0.17)
Q1 ≤ 1.0	48 (25.1)	9 (17.6)	1.0[a]	
Q2 (1.0–2.3)	48 (25.1)	9 (17.6)	1.00 (0.4–2.7)	
Q3 (2.3–4.6)	47 (24.6)	11 (21.6)	1.25 (0.5–3.3)	
Q4 (4.6–46.9)	48 (25.1)	21 (43.1)	2.44 (1.0–5.9)	
ESR1 (%)				0.10 (0.17)
Q1 ≤ 1.3	53 (25.0)	10 (16.9)	1.0[a]	
Q2 (1.3–2.1)	53 (25.0)	18 (30.5)	1.80 (0.8–4.3)	
Q3 (2.1–3.7)	53 (25.0)	9 (15.3)	0.90 (0.3–2.4)	
Q4 (3.7–95.4)	53 (25.0)	22 (37.3)	2.20 (1.0–5.1)	
GSTP1 (%)				0.30 (0.41)
Q1 ≤ 0.9	47 (25.3)	14 (25.5)	1.0[a]	
Q2 (0.9–1.3)	48 (25.8)	21 (38.2)	1.47 (0.7–3.2)	
Q3 (1.3–1.8)	44 (23.7)	10 (18.2)	0.76 (0.3–1.8)	
Q4 (1.8–50.0)	47 (25.3)	10 (18.2)	0.71 (0.3–1.8)	
HIN1 (%)				0.003 (0.02)
Q1 ≤ 0.7	33 (25.2)	8 (22.2)	1.0[a]	
Q2 (0.7–1.0)	33 (25.2)	10 (27.8)	1.25 (0.4–3.6)	
Q3 (1.0–2.0)	32 (24.4)	1 (2.8)	0.13 (0.0–1.1)	
Q4 (2.0–24.2)	33 (25.2)	17 (47.2)	2.13 (0.8–5.6)	
P16 (%)				0.41 (0.43)
Q1 ≤ 0.6	48 (25.4)	13 (24.5)	1.0[1]	
Q2 (0.6–1.0)	47 (24.9)	8 (15.1)	0.63 (0.2–1.7)	
Q3 (1.0–2.5)	47 (24.9)	15 (28.3)	1.18 (0.5–2.7)	
Q4 (2.5–39.9)	47 (24.9)	17 (32.1)	1.34 (0.6–3.1)	
RARβ (%)				0.65 (0.45)
Q1 ≤ 0.8	58 (26.1)	12 (20.0)	1.0[a]	
Q2 (0.8–1.2)	60 (27.0)	16 (26.7)	1.29 (0.6–3.0)	
Q3 (1.2–1.7)	48 (21.6)	17 (28.3)	1.71 (0.7–3.9)	
Q4 (1.7–14.2)	56 (25.2)	15 (25.0)	1.30 (0.6–3.0)	

[a]Reference category.

Table 4. Association between quartile levels of mean methylation scores and node-negative breast cancer (continued)

	BBD controls N %	BC cases negative nodes N %	BC cases negative nodes OR (95% CI)	p (q)
RASSF1 (%)				0.86 (0.49)
Q1 ≤ 1.1	51 (25.5)	11 (22.0)	1.0[a]	
Q2 (1.1–1.7)	49 (24.5)	13 (26.0)	1.00 (0.3–2.9)	
Q3 (1.7–2.9)	50 (25.0)	15 (30.0)	0.90 (0.6–3.3)	
Q4 (2.9–68.2)	50 (25.0)	11 (22.0)	1.02 (0.4–2.6)	
SFRP1 (%)				0.47 (0.43)
Q1 ≤ 0.6	46 (25.0)	10 (18.9)	1.0[a]	
Q2 (0.6–1.1)	46 (25.0)	12 (22.6)	1.20 (0.5–3.1)	
Q3 (1.1–4.5)	46 (25.0)	19 (35.8)	1.90 (0.8–4.5)	
Q4 (4.5–64.5)	46 (25.0)	12 (22.6)	1.20 (0.5–3.1)	
TWIST (%)				0.04 (0.14)
Q1 ≤ 2.3	50 (25.0)	5 (8.9)	1.0[a]	
Q2 (2.3–3.5)	51 (25.5)	19 (33.9)	3.73 (1.3–10.7)	
Q3 (3.5–4.9)	49 (24.5)	14 (25.0)	2.86 (1.0–8.5)	
Q4 (4.9 -15.4)	50 (25.0)	18 (32.1)	3.60 (1.2–10.4)	

[a]Reference category.

Table 5. Number and percent of study subjects with mean methylation scores of 5% or more for each gene among the three study groups

	BBD controls		BC cases negative nodes		BC cases positive nodes[a]		
	N	%	N	%	n	%	p[b]
APC	10	5.1	1	1.8	8	4.2	0.81
BRCA1	31	21.1	10	29.4	46	29.1	0.12
CCND2	11	6.4	4	7.8	20	11.2	0.13
CDH1	41	21.5	20	39.2	48	24.0	0.63
ESR1	48	22.6	18	30.5	52	26.0	0.49
GSTP1	11	5.9	3	5.5	17	9.6	0.24
HIN1	16	12.2	6	16.7	31	22.0	0.04
P16	29	15.3	6	11.3	33	17.1	0.68
RARβ	7	3.2	2	3.3	7	3.1	1.0
RASSF1	18	9.0	8	16.0	21	10.9	0.61
SFRP1	41	22.3	11	20.8	51	25.9	0.47
TWIST	49	24.5	18	32.1	71	37.9	0.004

[a]Includes cases with uncertain node status; [b]p value comparing node-positive BC cases to BBD controls.

suppressor genes (*APC*, *BRCA1*, *CDH1*, *CCND2*, *ESR1*, *GSTP1*, *HIN1*, *p16*, *RASSF1*, *RAR*β, *SFRP1* and *TWIST1*) frequently found to be methylated in breast cancers but not in normal breast tissue,[3] including several genes that have been observed to be methylated in the serum of breast cancer patients.[15] We found evidence that median levels of promoter methylation in serum DNA were modestly higher in breast cancer cases than benign breast disease controls, and higher in node-positive breast cancer cases than in node-negative cases. Further, analyses focused on the distribution of lymph node-positive cases vs. benign breast disease controls in each quartile level of gene promoter methylation found the most statistically significant results for *TWIST, SFRP, ESR1, P16, APC* and *HIN1* with p values ranging between 0.04 and 0.21. Additionally, we found the proportion of individuals with ≥ 5% methylation to be higher in lymph-node positive breast cancer cases than in benign breast disease control subjects for 10 of 12 genes. Despite the modestly higher methylation patterns we observed in cases compared with controls, our ability to discriminate between cases and controls was no better than random chance.

As reviewed by others,[14,19] a number of studies using primarily methyl-specific PCR or MethylLight assays have reported an association between serum DNA promoter methylation and breast cancer. However, most of these studies have had a small sample size, several have not included a control group, and the sensitivity estimates for individual genes have varied widely. Among the larger studies, Hoque and colleagues[15] reported a sensitivity of 62% and specificity of 87% with four candidate genes

(*APC*, *GSTP1*, *RASSF1A* and *RARβ2*) using quantitative methylation-specific PCR (QMSP) in a study of 47 breast cancer cases and 38 control subjects. In this study, Hoque and colleagues[15] reported that only 13% of the controls had methylation in any of these four tumor-related genes. By contrast, a more recent study by the same group[20] reported that nearly 50% of 157 cancer-free individuals had one or more of six tumor-related genes (*APC*, *GSTP*1, *RARβ2*, *CCND2*, *MGMT* and *P16*) methylated in serum using QMSP, including 22% in *RARβ2* alone. These latter two studies highlight the variability in percent methylation in serum DNA in patients and controls that has been observed across studies. A recent case-control study nested within a prospective cohort by Brooks and colleagues found no ability to discriminate breast cancer cases diagnosed in the prior six months (n = 50) from control subjects (n = 150) using the same gene panel as Hoque and colleagues.[15,16] The authors found that the methylation levels in breast cancer cases were less than expected but methylation levels in control subjects were higher than expected. Brooks and colleagues[16] utilized quantitative methylation specific PCR (QMSP) for the main analysis but reanalyzed a small subset of samples using pyrosequencing. Because pyrosequencing frequently did not confirm their methylation results as detected by QMSP, Brooks and colleagues[16] concluded that their main analysis contained a substantial number of false positives. Further, the overall null findings were attributed to non-specific amplification resulting in high-false positives, and the false negatives to the low amount of DNA template. In the present study, we employed pyrosequencing to examine methylation in serum in 12 genes yet still did not find any significant predictive ability based on methylation patterns.

There are several potential explanations for the low predictive ability we observed in our study. The first and most likely explanation is the lack of precision of the pyrosequencing assays. Indeed, the coefficients of variation (CVs) for the 0% methylation control sample across plates were poor, and even for the 50% methylation control sample we had less than desired CVs (8.5–34%).

Another potential explanation for the limited predictive power we observed is that the quality and/or quantity of the DNA in the samples could have been insufficient for accurately measuring methylation. For most of the genes we examined, however, the pyrosequencing pass rate ranged from 70% to 94%, suggesting that the DNA obtained was adequate for PCR amplification and sequencing. Still, the proportion of tumor DNA in serum may be too low to be detected reliably by pyrosequencing of bisulfite-modified DNA.[12,13] Zanetti-Dallenbach and colleagues[21] recently demonstrated that the amount of shed DNA in serum is ten times higher than in plasma, likely due to leaked white blood cells during the coagulation that occurs in the collection of serum. Thus, in the present analysis, white blood cell DNA could have diluted the methylation signal of the shed tumor DNA in serum.

Although it is also possible that promoter methylation status of another set of genes might more clearly distinguish between breast cancer cases and controls, the panel we selected was based on prior evidence that the included genes are frequently methylated in breast cancer tissue but not in normal tissue.[3,4] Furthermore, many of these genes have also been shown in several small studies to be more frequently methylated in the serum of breast cancer cases than that of controls.[14,15]

A special aspect in our study that deserves additional consideration is that all of the controls had a prior breast biopsy with evidence of benign breast disease. Some limited evidence indicates that increased DNA methylation of some tumor suppressor genes is detectable in benign breast disease.[22] The types of benign breast disease included in our study have only been minimally associated with breast cancer.[23] The large, well-matched control group is a strength of our study. If it were in fact true that our generally null findings reflect our choice of women with benign breast disease as controls, our findings would still imply that this panel of genes would not be useful in a clinical setting because benign breast disease is a common condition.

In summary, we were able to detect modest differences in promoter methylation in serum DNA in breast cancer cases and controls. Our findings and those of the recent study by Brooks and colleagues[16] emphasize the need to demonstrate the reliability and validity of the methods being proposed to measure low levels of promoter methylation in the small quantities of tumor-derived DNA in circulation, as well as the need to design sufficiently sized prospective studies with well-selected control groups.

Materials and Methods

Between 1977 and 1987, all patients about to undergo breast biopsy or mastectomy in three hospitals in Grand Rapids, Michigan were invited to provide serum as part of a study to assess new putative breast cancer markers. Participants provided written informed consent and completed an in-person interview assessing breast cancer risk factors. Information on pathology and extent of disease was abstracted from medical records. Serum was collected prior to the day of surgery or on the day of surgery for all study subjects. Before surgery, 5,358 volunteers donated 20 mL of non-fasting blood that was collected in sterile vacutainers, immediately chilled, and allowed to clot. The serum was separated within two hours and then divided into 1 mL aliquots and stored at -70°C in sealed glass vials. Specimens were then shipped frozen, in containers with dry ice, first to a central repository at the Mayo Foundation and subsequently to the National Cancer Institute and stored at -70°C to -85°C at both locations.[24]

For the present study, eligible women were postmenopausal, had no prior cancer or diabetes, and were not currently using hormone replacement therapy or oral contraceptives at the time of the blood draw. In addition, individuals had to have 2 mL or more of serum remaining in the bank. A total of 325 breast cancer cases (69 node-negative breast cancer cases and 256 primarily node positive breast cancer cases) and 249 control subjects were selected for this study. The mean time from the date of malignant diagnosis and blood collection was 0.46 d (95% CI: -0.7, 1.6) for node-negative cases and 0.4 d (95% CI: -3.0, 3.9) for node-positive cases. Because the cases were diagnosed in the 1970s, data on estrogen receptor, progesterone receptor and HER2/neu receptor status were not available. Control subjects had benign breast disease conditions that have been linked to no

or minimally increased risk of breast cancer.[23] Benign conditions included 71 women with non-proliferative benign changes (e.g., atrophic lobules, apocrine metaplasia) and 178 women who had low-risk proliferative conditions (e.g., cysts, fibroadenoma and mild hyperplasia).

Analysis of DNA methylation. DNA methylation analyses were conducted by EpigenDX Inc. Serum specimens were shipped on dry ice from the National Cancer Institute to EpigenDX Inc. The laboratory was blinded as to case-control status of the study subjects. DNA was extracted from 500 μL of serum and eluted in 30 μL of buffer; estimated to contain between 10 to 50 ng of DNA all of which was modified with bisulfite treatment (during which unmethylated cytosines are converted to uracil) and eluted in 18 to 20 μL of buffer. One microliter of bisulfite-modified DNA was used for each gene-specific amplification and 10 μl of the PCR products were sequenced by Pyrosequencing PSQ96 HS System (Qiagen). If the pyrosequencing failed due to low signal, a nested PCR reaction was done using the DNA from the initial PCR reaction and a second pyrosequencing was conducted. The methylation status of each locus (see **Table 2** for number of CpG sites per gene) was analyzed individually as a T/C SNP using QCpG software (Qiagen). Resulting pyrograms and percent methylation scores for each CpG site were received from EpigenDX, Inc. Each pyrogram was visually inspected for quality controls including bisulfite-conversion, expected sequence order and peak height. Data from only those pyrograms that passed all quality controls were included in the analyses. The pass rate ranged from 70% to 94%.

For the methylation analysis of each gene, serum samples from cases and control subjects were arranged randomly on eight 96-well plates (the same order of cases and controls for each gene). Due to technical laboratory issues, 36 study subjects on the first plate for each gene were omitted from all analyses. Thus, the final sample size consisted of 304 breast cancer cases (241 with primarily node-positive and 63 with node-negative disease) and 234 control subjects. Of the 241 primarily node-positive cases, 159 had at least one documented positive node, and the node status on the remaining 82 breast cancer cases was uncertain. Thus, the node-positive group may include some individuals who were node-negative. The mean time between the original date of blood collection and the methylation analysis was 29.4, 28.2 and 28.8 y for node-positive breast cancer cases, node negative breast cancer cases, and control subjects, respectively.

The number of CpG sites interrogated per gene varied from a low of 3 for *RAR*β to a high of 27 for *HIN1*. As displayed in **Table 2**, the percentage of subjects with available gene methylation data ranged from a low of 51.1, 58.5, and 56.0% for *HIN1* to 88.9, 92.5 and 94.9% for *RAR*β for node-positive breast cancer cases, node-negative breast cancer cases and benign breast disease control subjects, respectively.

As part of the quality control procedure, 0%, 50% and 100% methylated DNA samples (provided by the laboratory) were included on each plate. The median (range) of the gene-specific coefficient of variation (CV) among the replicate measurements of the 0% methylated DNA sample was 52.5% (12–139%). The median (range) of CV among the replicate measurements of the 50% DNA methylated sample was 27% (8.5–34%). Lastly, among the replicates of the 100% DNA methylated sample, the median (range) of CV was 3.9% (0.68–19%). For all genes, the replicate measurements of the 100% methylated sample resulted in the lowest CV.

Statistical analysis. Evaluation of the association between case-control status and the percent of subjects in each quartile of mean methylation levels for each gene was based on logistic regression models. Two separate sets of analyses were conducted to compare (1) the controls to the node-positive subjects and (2) the controls to the node-negative subjects. The mean methylation levels across the CpG sites in each gene were categorized into quartiles based on the distribution in the control subjects. Two-sided p values were calculated based on likelihood ratio tests. Univariate models as well as multivariable models adjusted for age were fit.

As several genes were evaluated in this study, adjustment for multiple comparisons was performed based on the q value procedure.[25] If all genes below a specific p value threshold are denoted as "true markers," the associated q value provides a measure of the proportion of false discoveries. This procedure is less conservative than traditional methods, such as the Bonferroni correction, and is more powerful in detecting true associations.

A multivariable analysis assessing the predictive power of the panel of genes was conducted using the Random Forests algorithm.[18] The classifier was trained by setting the number of trees to 10,000 and by considering a random subset of three genes at each split of every tree. The predictive power of the panel of genes was assessed based on the proportion of subjects that were correctly classified as cases or controls, among samples left out of the training process (out of bag samples). The predictive power of the panel of genes was assessed based on the proportion of subjects that were correctly classified as cases or controls (i.e., sensitivity and specificity) among samples left out of the training process (out of bag samples). A p value denoting the statistical significance of the Random Forests classifier was calculated by a permutation test—the subjects' case/control labels were randomly permuted 100 times and the distribution of the out of bag error rate was estimated. The p value corresponding to the Random Forests classifier was calculated as the proportion of random permutations in which the out of bag error rate was equal to or lower than the observed out-of-bag error rate.

Box plots showing the distribution of mean percent methylation levels for each of the 12 genes among the control, node-negative and node-positive subjects are included in the **Supplemental Material**. The lines at the bottom, middle and top of each box denote the 25th, 50th (median) and 75th percentiles of each distribution. Circles represent outliers in both tails of the distribution.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/22220

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