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14. ABSTRACT

DNA methylation is increasingly recognized as an early molecular change in benign epithelium that is implicated in the development of high risk preneoplasia and cancer. Using a genome-wide screen we have previously identified methylation markers optimized for RP-FNA samples with potential for tissue-based breast cancer risk stratification. Most methylation markers are most relevant to ER-POS breast cancer. We have thoroughly assessed 12 new markers in an archival RP-FNA and cancer FNA sample set derived from 180 women. VCAN was identified as a gene preferentially methylated in ER-POS breast cancer and IRF7 as a gene preferentially methylated in ER-NEG breast cancer. We have now assembled a 7 marker panel with good coverage of ER-POS and ER-NEG breast cancer for assessment in prospectively acquired RP-FNA and cancer FNA samples. In addition, validation of an RP-FNA-based approach to risk stratification has been hampered by the lack of an unselected unaffected control cohort. We are rapidly expanding a community-based RP-FNA sample repository that provides an ideal unselected unaffected control cohort for validation of our panel in the coming year.

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Table of Contents

Page

INTRODUCTION

Hundreds of genes have been identified that show promoter region methylation in breast cancer. Some of these methylation changes will be integral to the malignant phenotype while others likely reflect innocuous heterogeneity within tumor cell populations. The classical approach for recognizing cancer-relevant methylation changes has been to identify promoter region methylation that leads to silencing of genes whose expression is known to resist malignant transformation (i.e. tumor suppressor genes). We propose a different approach based on quantitative assessment of DNA methylation for specific genes in large numbers of benign breast epithelial cell samples and cancers. Our approach is based on the observation that DNA methylation is the earliest molecular change observed during malignant transformation. We hypothesize that this is due to expansion of a minor cell population already resident in benign breast tissue. If the specific genes, whose promoter region methylation characterizes this population, could be identified then it may be possible to develop a tissue-based breast cancer risk stratification test based on the size of this cancer-progenitor cell population. The criteria we have established for advancing markers with the potential to recognize a cancer progenitor cell population in benign breast samples are: 1) identifies a small population of cells in most benign samples with evidence for marked expansion of this population in cancer samples, 2) presence in a benign sample predicts presence in an associated cancer from the same individual, and 3) occurs with a greater frequency in benign samples from women who have recently been diagnosed with a primary breast cancer as compared to age-matched women without breast cancer.

BODY

Task 1: Assess 7 new markers with the potential to improve risk prediction for ER(-) breast cancer in African-American women.

Our initial DoD grant used unbiased whole genome and candidate gene approaches to identify 284 breast cancer methylation markers. We reduced this list to 63 genes by eliminating those that were methylated in lymphocytes (this would interfere with a clinical test based on random periareolar fine needle aspiration [RP-FNA] samples), and those that were generally methylated in all epithelial cells using a comparatively low sensitivity assay (MSP). We have previously thoroughly evaluated 17 of these genes and one aim of this extension is to thoroughly evaluate additional genes.

We completed QM-MSP assays for 12 new markers in 180 archival samples. A detailed description of the data analysis is provided in the SUPPORTING DATA section. One important finding is the recognition of IRF7 as a gene that is disproportionately methylated in ER-NEG breast cancer and in breast cancer from African-American women.

FINAL GENE LIST

Based on all available data from the initial funding period and new data generated with this extension we have selected the following 7 genes for assessment in the additional tasks listed below: RASSF1A, CCND2, GNE, PSAT1, HS3ST2, PECI, VCAN, IRF7. These genes provide good coverage of ER-POS and ER-NEG breast cancer and have some potential for risk stratification when assessed in benign RP-FNA samples.

Task 2: Determine whether expression of CLDN1 and CPNE8 (and any genes advanced from Task 1 lacking this documentation) is regulated by promoter region hypermethylation (Months 1 – 9).

CLDN1 and CPNE8 did not make our final gene list so regulation of their expression by promoter region methylation will not be explored. Regulation of gene expression by promoter region methylation is well described for RASSF1A, CCND2, PSAT1, HS3ST2, VCAN, and IRF7 so additional work is not needed.

PECI (AKA ECI2) is a monofunctional peroxisomal Delta(3),Delta(2)-enoyl-CoA isomerase. Very little is known about this gene and it has not previously been associated with breast cancer. We observed a very strong methylation signal for PECI by MSP in two breast cancer cell lines, but not lymphocytes or human mammary epithelial cultures. We observed only low levels of methylation by QM-MSP in primary breast cancers. Most, striking, however, was the observation that PECI is frequently methylated in benign RP-FNA samples from patients with ER-NEG breast cancer. We would like to pursue this gene further, with redesign of the QM-MSP primers and probes and documentation of regulation of gene expression by promoter region methylation.

Task 3: Generate data concerning the performance characteristics of the RP-FNA epigenetic testing in archival samples (Months 6 – 21).

The final 7 marker panel for this task has been defined (Final Gene List, above). We are currently optimizing a QM-MSP assay that includes these 7 genes. We are assembling the required sample sets at UTSW and also working with Dr. Carol Fabian to have samples sent from Kansas University. Per our 2012 Q1 report, despite providing a detailed letter of collaboration for the grant application, one of our collaborators, Victoria Seewaldt at Duke University, expressed concern about sharing any of her samples when we contacted her after receiving notification of funding. She has since stopped responding to emails or phone calls so the protocol was modified to exclude her site.

Task 4: Initiate a prospective epigenetic testing registry for unaffected women.

Despite receiving local IRB approval on 10/25/2011, we did not receive our final DoD HPRO approval until 5/18/2012. We immediately arranged for our collaborators, Cooper Research Institute and Southern Methodist University, to mail invitation letters. Per our 2012 Q2 report, we encounter unanticipated institutional obstacles to performing the RP-FNA sampling on community volunteers in our Breast Center procedure room. New policies and procedures had to be established, and we needed to obtain buy-in from the clinic staff and administrators. This was all navigated successfully and the response from the community has been phenomenal. To date we have received more than 115 calls from prospective participants and have contacted 77. We are able to schedule 2 – 4 sampling visits every Monday and Friday for the foreseeable future. We currently have as many volunteers as we can manage and are staging the mailing of additional letters so that we can quickly respond to phone calls. To date we have sampled 34 women. More than 90% of the participants have scored the discomfort of the procedure as 2 out of 10. There have been no serious adverse events. The experience of the participants has been uniformly positive and many have referred their friends for participation.

KEY RESEARCH ACCOMPLISHMENTS

- We have identified additional methylation markers that are specifically associated with ER-POS (VCAN) or ER-NEG (IRF7)breast cancer.
- We have identified a methylation marker that is associated with breast cancer in African-American women (IRF7).
- We have defined a 7-marker methylation panel that appears to have potential for tissue-based breast cancer risk stratification.
- We are rapidly expanding a community-based RP-FNA sample repository that can be used as an unselected unaffected control group for the prospective validation component of this application.

REPORTABLE OUTCOMES

We have not yet submitted any abstracts or manuscripts based on this work.

CONCLUSION

Most methylation markers are most relevant to ER-POS breast cancer. We have thoroughly assessed 12 new markers in an archival RP-FNA and cancer FNA sample set derived from 180 women. VCAN was identified as a gene preferentially methylated in ER-POS breast cancer and IRF7 as a gene preferentially methylated in ER-NEG breast cancer. We have now assembled a 7 marker panel with good coverage of ER-POS and ER-NEG breast cancer for assessment in prospectively acquired RP-FNA and cancer FNA samples. In addition, validation of an RP-FNA-based approach to risk stratification has been hampered by the lack of an unselected unaffected control cohort. We are rapidly expanding a community-based RP-FNA sample repository that provides an ideal unselected unaffected control cohort for validation of our panel on the coming year.

So What

Mathematical models are commonly used for breast cancer risk assessment, but even the best models suffer from very poor discrimination. That is, they are unable to reliably distinguish between women who will develop breast cancer and women who will not. This limits their clinical utility. We are working towards individualized tissue-based breast cancer risk stratification based on assessment of epigenetic changes in benign breast cells. Through the initial funding period, and work completed in the first year of this extension, we have assembled a 7-gene panel that may have value for epigenetic-based breast cancer risk stratification. We are currently making excellent progress towards prospectively collecting the samples required for an independent validation. The value of a prospectively acquired, community-based unselected, unaffected control cohort cannot be over stated.

REFERENCES

None

APPENDICES

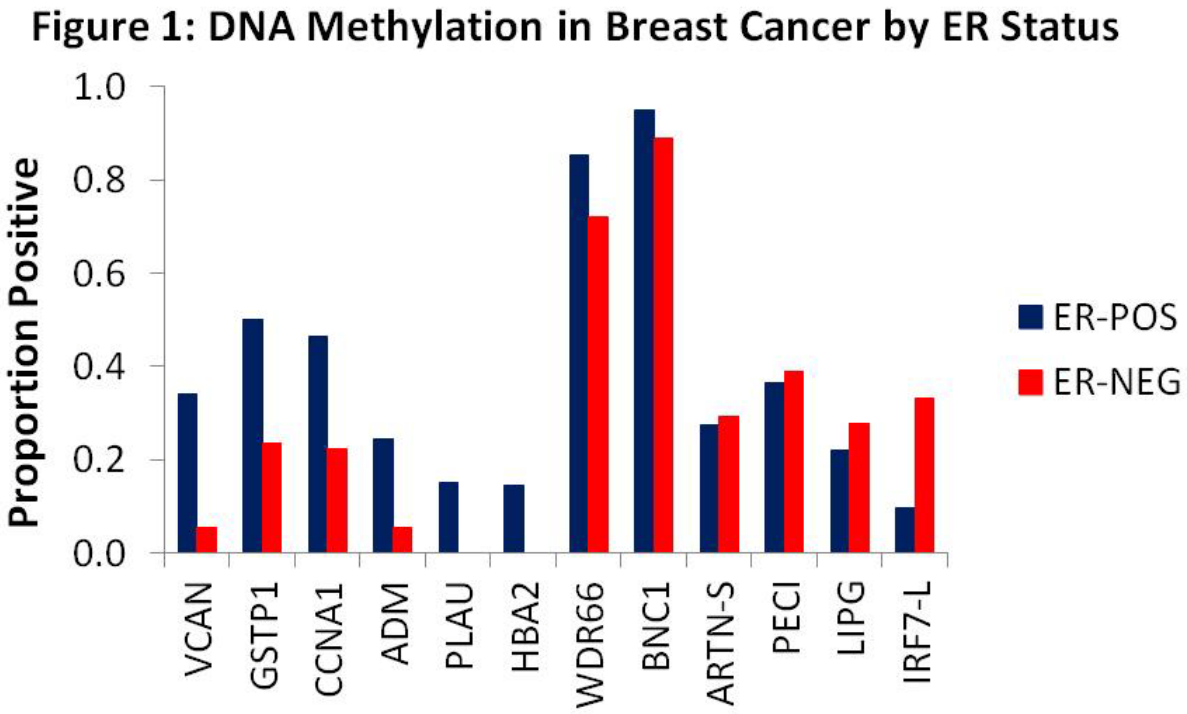
None

SUPPORTING DATA

1. DNA Methylation in Breast Cancer by Estrogen Receptor Status
We had previously assessed DNA methylation for 57 genes in a small panel of primary breast cancer samples (N=15). This small dataset suggested that some genes were preferentially methylated in ER-POS breast cancer and some in ER-NEG breast cancer. This aim was designed to validate these observations in a larger panel of breast cancer samples (N = 60).

The genes selected for this validation were:
ER-POS: GSTP1, HBA2, BNC1, and WDR66
ER-NEG: IRF7, PECI, ARTN, VCAN, ADM, LIPG, and PLAU

Figure 1 shows the proportion of ER-POS and ER-NEG breast cancers methylated for each gene.

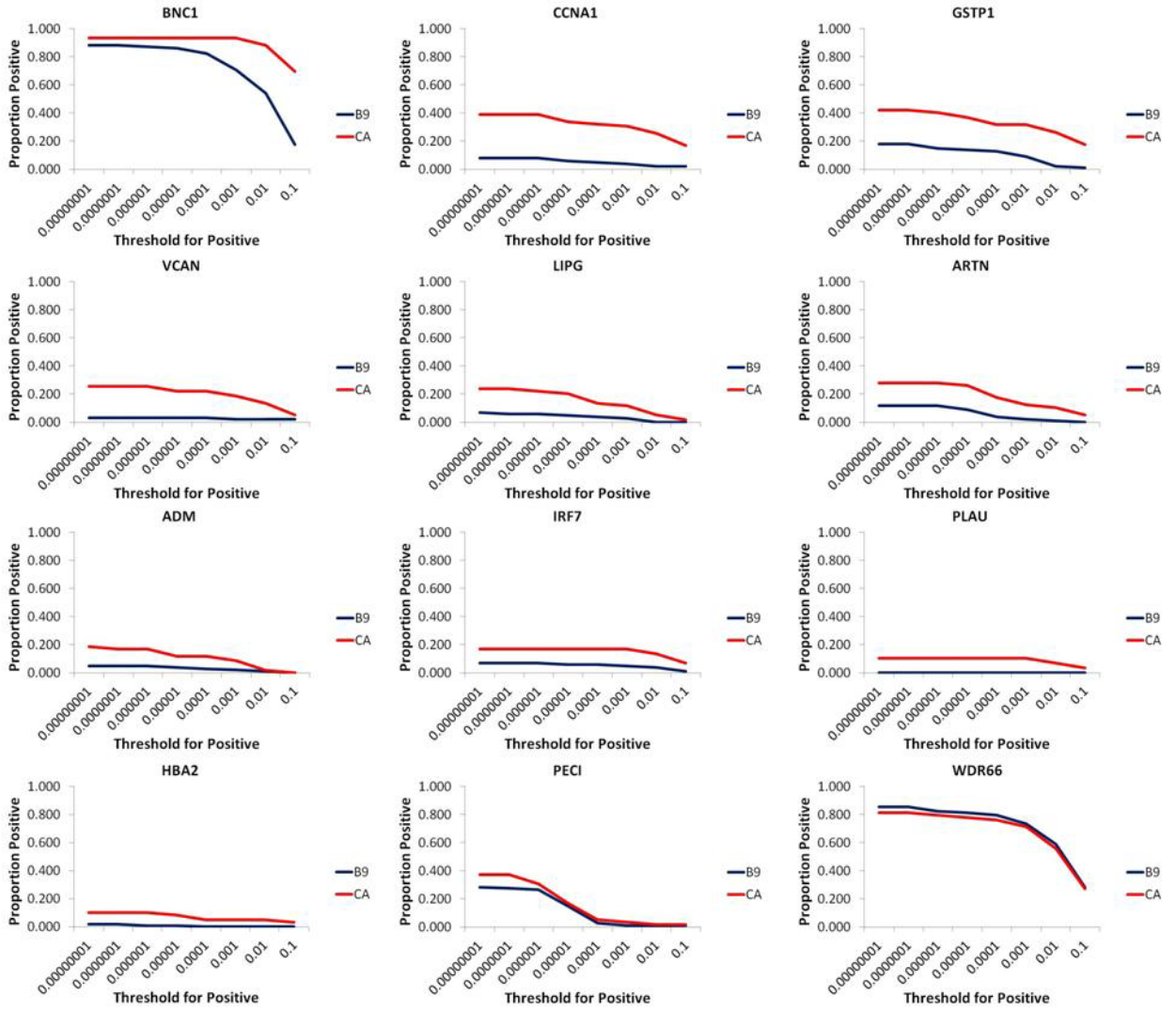


Interpretation: Six of the genes showed preferential methylation in ER-POS breast cancer, 5 showed an equivalent distribution between ER-POS and ER-NEG breast cancer and 1 showed preferential methylation in ER-NEG breast cancer. Statistically significant differences were found for the ER-POS-associated gene VCAN (P = 0.024) and for the ER-NEG-associated gene IRF7 (P = 0.05).

2. Breast Cancer Relevance of the New Methylation Markers

Promoter region methylation of the 12 genes described above was quantified by QM-MSP in 60 primary breast cancer samples and 113 benign RP-FNA samples. The proportion of samples testing positive for methylation across a range of threshold levels was compared between groups (Figure 2).

Figure 2: DNA Methylation in Cancers as Compared to Benign RP-FNA Samples



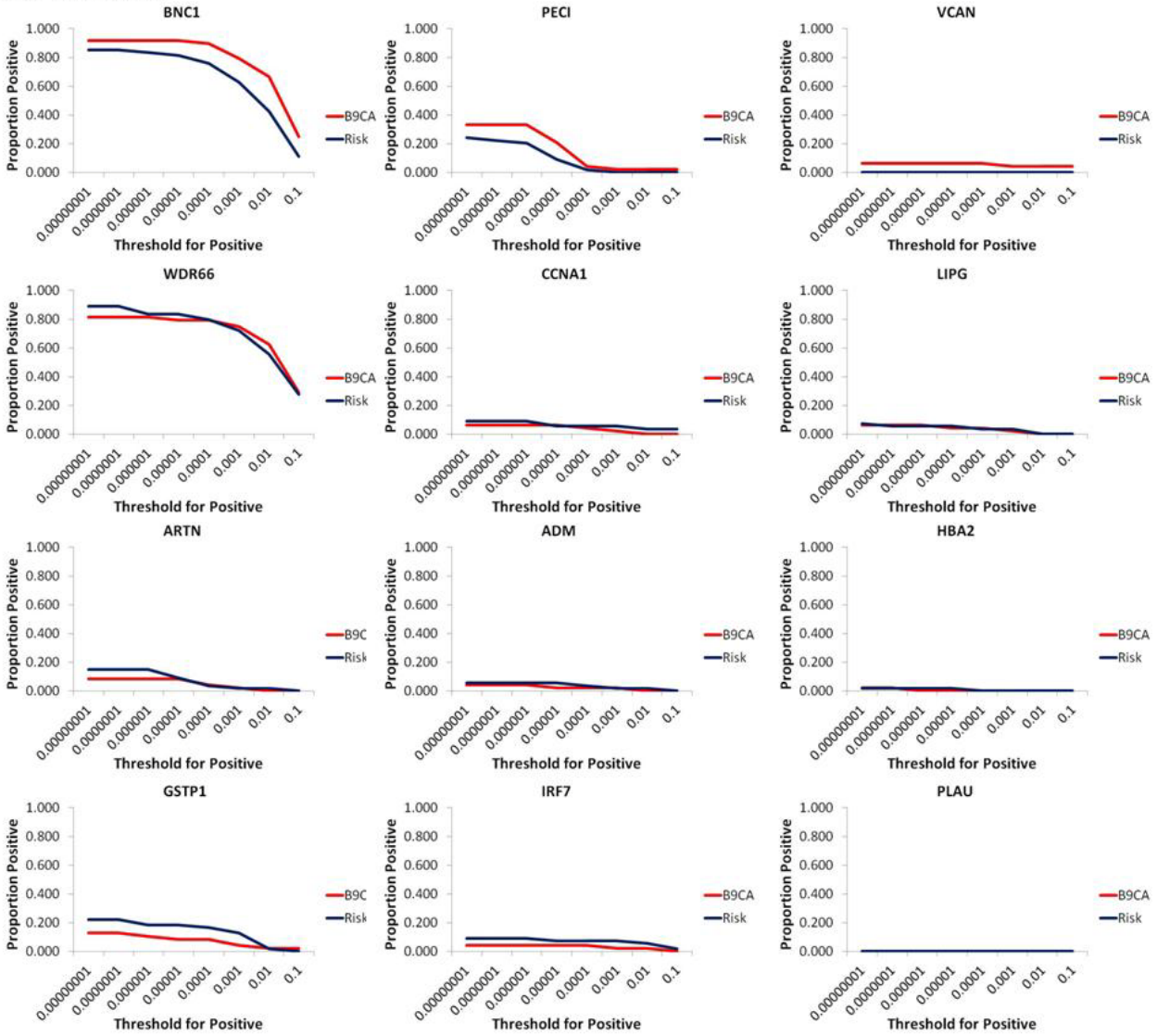
Interpretation: These plots provide a sense of the proportion of cells in a sample with promoter region methylation of a given gene (X-axis) and the proportion of individuals with methylation at that level (Y-axis). For instance, most individuals have small populations of BNC1-methylated cells in their benign RP-FNA samples, while most breast cancers show marked expansion of this population. Each of the markers, except PECI and WDR66 showed low level methylation in a higher proportion of cancer samples than benign RP-FNA samples. Most of these markers appear to be identifying a minor cell population occurring in a small proportion of benign samples and a somewhat greater proportion of cancers. This pattern is consistent with methylation that is acquired after transformation, but

maintained in only a small fraction of the tumor cells. Only BNC1, CCNA1, and GSTP1 show noticeable expansion of the methylated population in cancer.

DNA Methylation in Benign RP-FNA Samples in Relation to Breast Cancer Risk

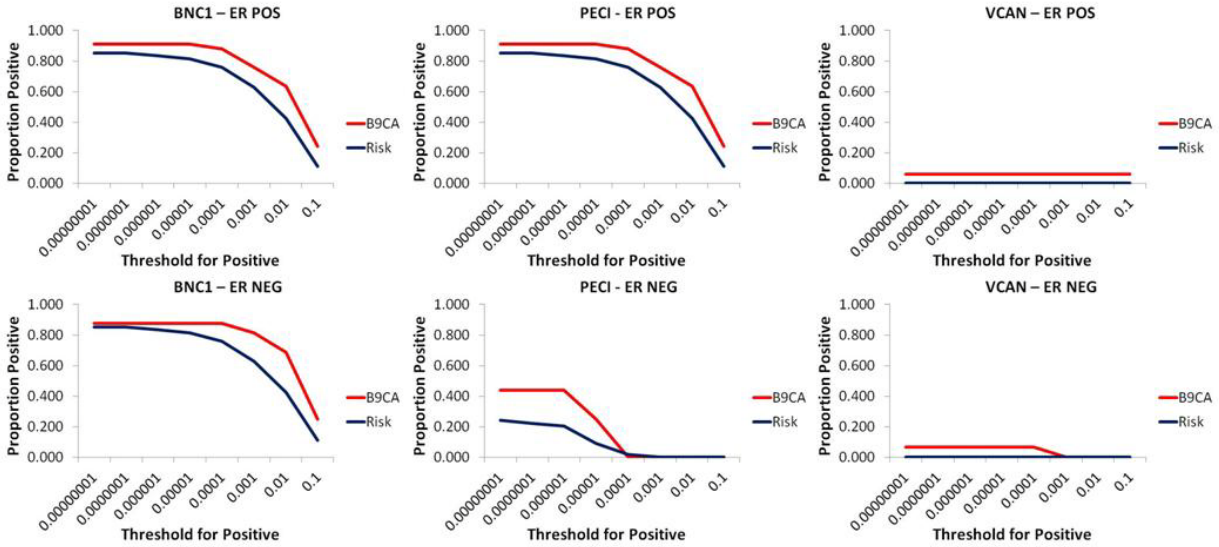
Our working hypothesis is that benign breast cells from women recently diagnosed with a primary breast cancer will exhibit high risk molecular alterations at a greater frequency than benign cells from age-matched women who have not been diagnosed with breast cancer. The 12 genes tested in breast cancer samples (described above) were also assessed by QM-MSP in benign RP-FNA samples from 59 breast cancer patients (B9CA)and 54 unaffected women (RISK). Figure 3 shows plots of the proportion of cases positive for methylation for each group across a range of threshold values. Values for the B9CA curve will be higher than those for the RISK curve across a wide range of threshold values for markers with some potential for risk stratification.

Figure 3a: DNA Methylation in Benign RP-FNA Samples from Breast Cancer Patients (B9CA) and Unaffected Women (RISK) . All Samples



The B9CA samples were divided into two subgroups based on the ER expression of the associated cancer and the analysis repeated.

Figure 3b: DNA Methylation in Benign RP-FNA Samples from Breast Cancer Patients (B9CA) and Unaffected Women (RISK) . According to the ER status of the cancers associated with the B9CA samples.



Interpretation: BNC1, PECI and VCAN showed low level methylation in a greater proportion of benign RP-FNA samples from cancer patients than samples from age-matched unaffected women. There was evidence for modest expansion of the BNC1-methylated cell population in women who developed ER-POS or ER-NEG breast cancer, and expansion of the PECI-methylated and VCAN-methylated cell population in women who developed ER-POS but not ER-NEG breast cancer.

Table 1: P-values for Differences in Methylation of Benign Samples Between Age Groups

Gene	[a]Kruskal-Wallis Test on Means	[b]Chi-Square for Trend
WDR66	0.066	0.077
BNC1	0.830	0.827
PECI	0.374	0.966
ADM	0.820	0.609
ARTN	0.192	0.138
GSTP1	0.218	0.070
LIPG	0.090	0.011
CCNA1	0.655	0.428
VCAN	0.301	0.128
IRF7	0.483	0.496
HBA2	[c]ND	ND
PLAU	ND	ND

[a]Non-parametric comparison of mean methylation fraction by age group.
[b]Chi-suare for trend on proportion of samples with any methylation signal by age group.

[c]Not Done. Too few positives.

Age Acquired Methylation (Benign Samples)

We have previously observed that methylation frequency for some genes increases with age. This can confound efforts to identify breast-cancer risk-associated markers because breast cancer incidence also increases with increasing age. When assembling this training sample set we were very careful to match the ages for benign samples from breast cancer patients and unaffected women. This matching was successful based on the mean age of 54 for the breast cancer patients and 55 for the unaffected women (P = 0.603). An F-test confirmed that the variance was nearly identical for these two groups (P=0.994).

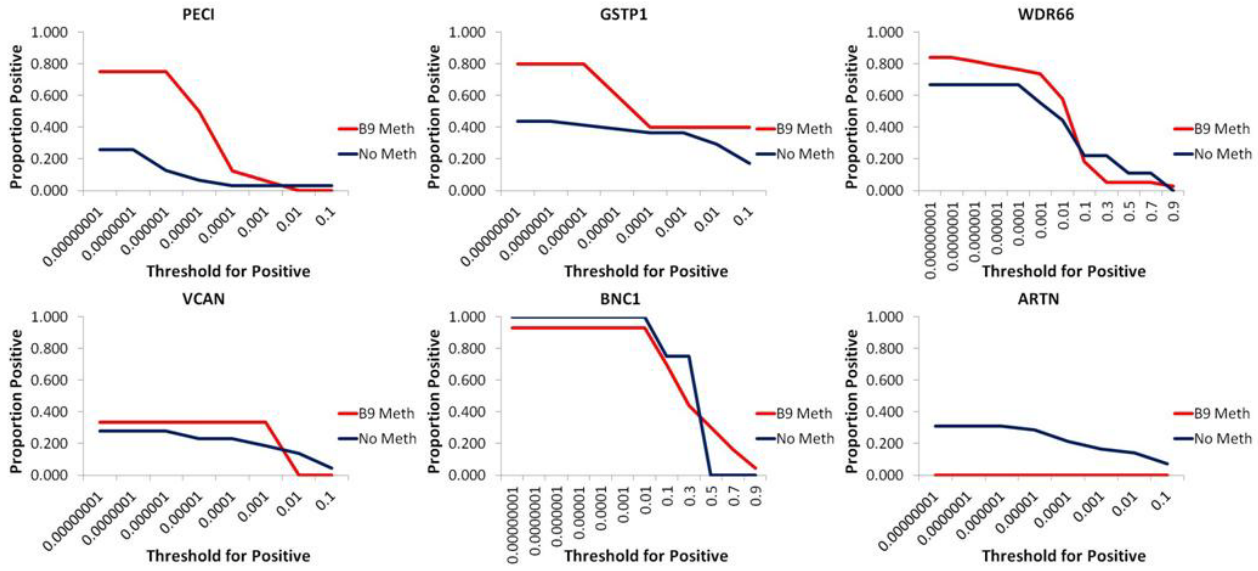
For each of the 12 markers evaluated in this training set, we assessed age association by comparing mean methylation fractions and proportion of samples with any methylation signal for 113 benign RP-FNA samples divided into three age groups: 40 – 49 (N = 32), 50 – 59 (N = 54), and ≥ 60 (N=27).

Interpretation: WDR66, a marker we have classified as "not relevant to breast cancer" does show a trend for increasing methylation with increasing age. LIPG showed a statistically significant loss of methylation with increasing age (proportion positive = 17%, 4% and 0% for the three age groups defined above. P = 0.011). GSTP1 showed a similar trend (27%, 17%, and 8%. P = 0.07).

Methylation of the Same Gene in Cancer and Paired Benign Samples

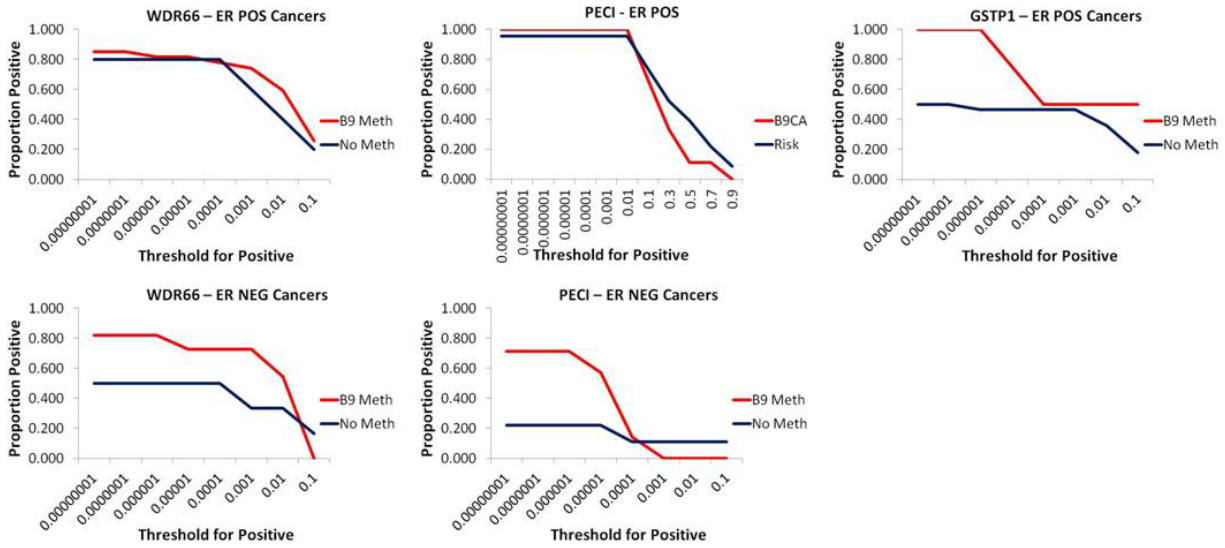
If DNA methylation of specific genes is identifying and quantifying the pool of breast epithelial cells primed for malignant transformation then methylation of that gene should occur more frequently in an associated cancer if it is also present in the paired benign sample. We assessed methylation of 12 genes in 58 paired cancer and benign RP-FNA samples. Figure 4 shows the proportion of cancer samples showing methylation for a given gene (across a range of thresholds) when that gene was also methylated in the paired benign sample (B9 Meth) or not (No Meth). There were too few benign samples with methylation of HBA2, PLAU, ADM, LIPG, VCAN, or IRF7 to permit this type of analysis.

Figure 4a: Methylation in Paired Benign and Cancer Samples According to Methylation Status of the Benign Sample. ER POS and ER NEG Combined



The B9CA samples were divided into two subgroups based on the ER expression of the associated cancer and the analysis repeated.

Figure 4b: DNA Methylation in Benign RP-FNA Samples from Breast Cancer Patients (B9CA) and Unaffected Women (RISK) . According to the ER status of the cancers associated with the B9CA samples.

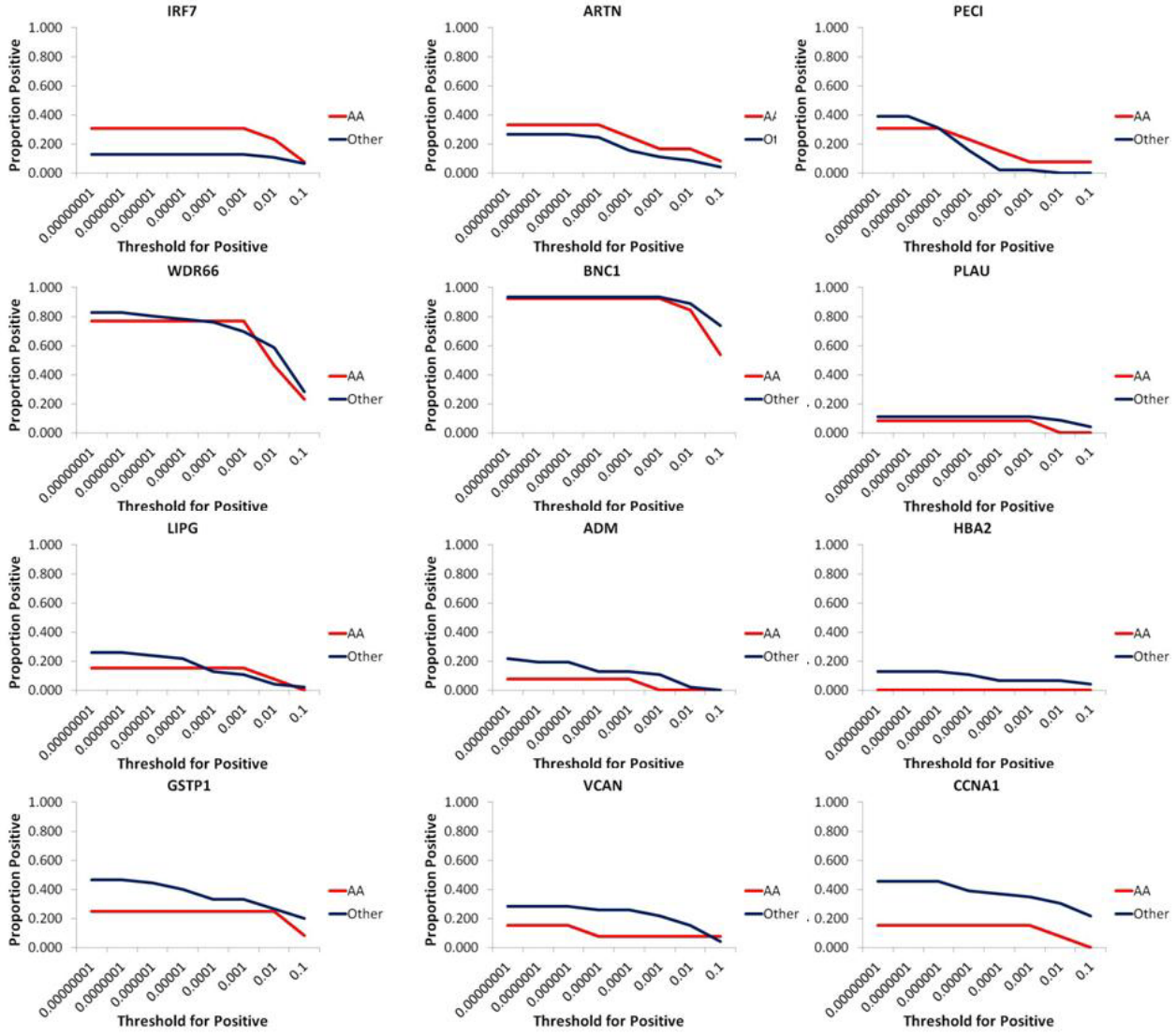


Interpretation: Methylation of PECI or GSTP1 in a benign RP-FNA sample predicted methylation in the associated breast cancer. For PECI, the association was only apparent in women who developed ER-NEG breast cancer and was most consistent with persistence of a minor cell population rather than expansion of the PECI-methylated cell population. There were too few benign RP-FNA samples from ER-NEG cancer patients with detectable methylation of GSTP1 to permit an analysis, but the data suggest persistence of a minor population of GSTP1-methylated cells in ER-POS cancers with expansion of this population. WDR66 did not appear to be cancer-relevant on the initial analysis that combined ER-POS and ER-NEG cancers, but methylation of WDR66 in a benign RP-FNA sample from a ER-NEG cancer patient did predict WDR66 methylation in the associated cancer.

Methylation in Breast Cancer by African-American (AA) Race

Our previous work with 17 methylation markers consistently showed higher levels of methylation and more frequent methylation in breast cancers from Caucasian women than African-American woman. An exception was GNE which showed essentially equivalent methylation in Caucasian and African-American women. One aim of the current study was to compare methylation levels and rates between breast cancers from African-American and Caucasian women. Figure 5 shows the proportion of cancer samples showing methylation for a given gene (across a range of thresholds) according to the patient's race (African-American or other).

Figure 5: DNA Methylation in Breast Cancer by African-American (AA) of Other Race



Interpretation: ADM, HBA2, GSTP1, VCAN, and CCNA1 showed the same pattern we have observed previously with more frequent methylation observed among Caucasian women. Notably, below the 0.1 threshold, cancers from African-American women showed more frequent methylation of IRF7 than cancers from Caucasian women. This gene was also more frequently methylated in ER-negative breast cancer than ER-positive breast cancer (Figure 1). ARTN and PECI are also of interest. ARTN because it is clearly methylated at the same or higher frequency in cancers from African-American women as compared to Caucasian women, and PECI because higher levels of methylation (>0.1) were only observed in a small subset of African-American women and not in Caucasian women.