Published in final edited form as:
Cancer Epidemiol. 2010 December ; 34(6): 717–723. doi:10.1016/j.canep.2010.05.006.

DNA Methylation in Pre-Diagnostic Serum Samples of Breast Cancer Cases: Results of a Nested Case-Control Study

Jennifer D. Brooks[a,b], **Paul Cairns**[c], **Roy E. Shore**[a,d], **Catherine B. Klein**[e], **Isaac Wirgin**[e], **Yelena Afanasyeva**[a], and **Anne Zeleniuch-Jacquotte**[a,f]

[a]Division of Epidemiology, Department of Environmental Medicine, New York University School of Medicine, New York, NY 10016

[b]Department of Epidemiology and Biostatistics, Memorial Sloan-Kettering Cancer Center, New York, New York, 10065

[c]Department of Surgical Oncology and Pathology, Fox Chase Cancer Center, Philadelphia, PA 19111

[d]Radiation Effects Research Foundation, 5-2 Hijiyama Koen, Minami-ku, Hiroshima 732-0815, Japan

[e]Molecular Toxicology and Carcinogenesis Program, Department of Environmental Medicine, New York University School of Medicine, Tuxedo, NY 10987

[f]New York University Cancer Institute, New York University School of Medicine, New York, NY 10016

Abstract

Promoter methylation of tumor-suppressor genes is a frequent and early event in breast carcinogenesis. Paired tumor tissue and serum samples from women with breast cancer show that promoter methylation is detectable in both sample types, with good concordance. This suggests the potential for these serum markers to be used for breast cancer detection.

The current study was a case-control study nested within the prospective New York University Women's Health Study cohort aimed to assess the ability of promoter methylation in serum to detect pre-clinical disease. Cases were women with blood samples collected within the six months preceding breast cancer diagnosis (n=50). Each case was matched to 2 healthy cancer-free controls and 1 cancer-free control with a history of benign breast disease (BBD).

Promoter methylation analysis of four cancer-related genes: — *RASSF1A*, *GSTP1*, *APC* and *RARβ2*, — was conducted using quantitative methylation specific PCR. Results showed that the frequency of methylation was lower than expected among cases and higher than expected among controls. Methylation was detected in the promoter region of: *RASSF1A* in 22.0%, 22.9% and 17.2% of cases, BBD controls and healthy controls respectively; *GSTP1* in 4%, 10.4% and 7.1%

Corresponding Author and Request for Reprints: Dr. Jennifer Brooks Memorial Sloan-Kettering Cancer Center Department of Epidemiology and Biostatistics 307 East 63rd Street, 3rd Floor New York, NY 10065 **Phone:** 1-646-735-8068 **Fax:** 1-646-735-0012 brooksj@mskcc.org.

Conflict of Interest Statement: P.C. is a paid consultant to Oncomethylome Sciences. The terms of this arrangement are being managed by Fox Chase Cancer Center in accordance with its conflict of interest policies.

respectively; *APC* in 2.0%, 4.4% and 4.2% respectively and *RARβ2* in 6.7%, 2.3% and 1.1% respectively.

Methylation status of the four genes included in this study was unable to distinguish between cases and either control group. This study highlights some methodological issues to be addressed in planning prospective studies to evaluate methylation markers as diagnostic biomarkers.

Keywords

DNA methylation; Breast Cancer; Early Detection; Serum; QMSP

Introduction

Routine screening with mammography has demonstrated that early detection leads to reduced breast cancer mortality [1], and the majority of women in the U.S. over the age of 40 undergo annual or biannual mammography [2-3]. There are, however, subgroups of women for whom mammography is less sensitive, e.g. women who are young, Asian, on hormone replacement therapy, and/or have dense breasts. Sensitivity in these groups of women has been estimated to be as low as 25% in women on hormone replacement therapy [4-7]. Mammography is also less sensitive for the detection of invasive lobular carcinomas (ILC) (sensitivity ranging between 57 and 81%) and small or diffuse tumors [8-9]. Specificity is also an issue, with one in two women who receive yearly mammograms expected to have at least one false positive result in her lifetime, leading to unnecessary biopsies and anxiety [10-11]. This indicates that a screening tool that could be used alongside mammography in women for whom it is less effective, could lead to improved screening practices. Such a tool would ideally be low cost and minimally invasive.

Silencing of tumor suppressor genes through the hypermethylation of their promoter regions has been shown to be a frequent event in carcinogenesis [12]. A large number of studies have shown the frequent methylation of genes involved in cell cycle regulation (*p16^{INK4A}*, *p14^{ARF}*, *p15*, *CCDN2*, *DAPK)*, DNA repair (*MGMT*, *hMLH1*), xenobiotic metabolism (*GSTP1*), signal transduction (*RARβ2*, *APC*, *ERβ*) and adhesion and metastasis (*CDH1*, *CDH13*) in breast cancer tumors [13-19]. These modifications are believed to occur early in the development of cancer [12], suggesting that aberrant DNA methylation could be a useful early biomarker of disease.

Tumor DNA is present in the circulation of people with cancer [20]. The mechanism of DNA release into circulation is poorly understood, but it is believed that DNA is released during tumor necrosis and apoptosis [21]. Hypermethylation of gene promoters has been detected in the serum/plasma DNA of breast cancer cases [22-25]. Furthermore, results from matched tumor and blood samples from the same patient have shown good concordance [26-30]. Because the drawing of blood has the advantage of being a minimally invasive procedure, it is of interest to assess the potential of hypermethylation of circulating DNA as an early detection marker of breast cancer.

The objective of this study was to assess the ability of the promoter methylation of a four-gene panel (*RASSF1A*, *GSTP1*, *APC* and *RARβ2*) in banked serum DNA to detect preclinical disease. The four genes included in our study were selected because they have known functions in carcinogenesis and have previously been shown to be frequently methylated in the tumors and serum/plasma of women with breast cancer [13-15,18,31]. We conducted a nested case-control study focusing on cases diagnosed shortly after blood donation (within 6 months) and matched controls from the same cohort of women.

Study Design

The New York University Women's Health Study (NYUWHS) Population

A cohort of 14 274 healthy women between the ages of 35 and 65 were enrolled in the NYUWHS between 1985 and 1991 at a breast cancer screening clinic [32]. Eligibility was restricted to those women who had not used hormonal medications or been pregnant in the six months prior to recruitment. At the baseline recruitment visit, women completed a self-administered questionnaire eliciting information about anthropometric variables, demographics, medical and reproductive history and lifestyle factors as well as a semi-quantitative food frequency questionnaire. Also at recruitment, 30 ml of non-fasting, peripheral venous blood was collected (using red-cap Vacutainer tubes) from all study participants. Serum was isolated and stored in 1 ml aliquots at −80°C until use.

Follow-up was conducted through the screening clinic when subjects returned for subsequent yearly visits (after baseline) until 1991. Since then, participants continue to be followed-up with self-administered questionnaires mailed every 3-4 years to update information on disease status, medical and reproductive histories and lifestyle factors. Reported cancer cases are confirmed through examination of medical reports and active follow-up is supplemented by linkage to the National Death Index and the state tumor registries of New York, New Jersey and Florida, in which most of the women reside. A capture-recapture analysis has indicated a breast cancer case ascertainment rate of 95% in this cohort [33]. Tumor stage and receptor status were obtained by reviewing medical and pathology reports. Histological subtypes were coded using the modified ICD-O codes (International Classification of Diseases, 9^{th} revision).

Nested Case-Control Study Design

A case-control study nested within the NYUWHS cohort was conducted. This study design uses pre-diagnostic samples which is essential to assess the ability of any biomarker to detect pre-clinical disease. Breast cancer cases in women for whom serum samples had been collected within the 6 months prior to breast cancer diagnosis were included. For each case, two sets of controls were selected. In the first set, two healthy controls for each case were selected at random from matched sets of women who were alive and free of any cancer and who had no history of benign breast disease (BBD). In the second set, one control subject was randomly selected among matched sets of healthy cancer-free women with a history of BBD. To ensure that controls were truly free of cancer at the time of blood donation, this study required controls to be cancer-free for the duration of follow-up (median follow-up time 20.1 years, range 11.0-22.6 years). Individual matching on age (± 6 months) at, and date of, blood donation (± 6 months) was used to control for the potential effects of age and duration of sample storage on DNA methylation patterns. All procedures were approved by the New York University School of Medicine Institutional Review Board (IRB).

Methods

Quality Control Procedures

For all procedures, all four samples from a matched set (one case, two healthy controls and one control with BBD) were always analyzed on the same day and in the same batch to control for possible inter-batch measurement variation. The samples were blinded as to their case or control status.

DNA Isolation and Sodium Bisulfite Treatment

DNA was isolated from 1 ml aliquots of serum (never previously thawed) using the QIAamp DNA Blood Midi Kits (Qiagen, Valencia CA) as described by the manufacturer. Isolated

DNA was stored in 45 μl aliquots (the amount required for sodium bisulfite treatment) at −80°C to eliminate any unnecessary freeze-thaw cycles.

Chemical modification of isolated DNA with sodium bisulfite converts unmethylated cytosine to uracil while leaving methylated cytosine unchanged. This leads to the generation of detectable methylation-specific sequence variation. Sodium bisulfite conversion was conducted using the Qiagen Epitect bisulfite conversion kit (Qiagen, Valencia CA) as described by the manufacturer. Standards and samples to be included on the same PCR plate on a given day were bisulfite-treated in the same batch. Usually, the methylation analysis occurred on the same day as sodium bisulfite conversion to eliminate the effects of storage on the bisulfite-converted DNA.

Quantitative Methylation Specific PCR (QMSP)

Bisulfite treated DNA was analyzed using quantitative fluorescence-based real-time PCR (QMSP) as described by Eads et al [34]. Methylation-specific primers were designed to recognize the sequence of the methylated, bisulfite-treated gene region of interest. A second set of primers for β-actin (*ACTB*), were run in parallel and used as a control to normalize for DNA input. Primer and probe sequences were obtained from previous publications [23,35-36] and fluorescence was detected using the AB 7300 (Applied Biosystems, Foster City CA).

Each assay included standard curves using fully methylated DNA (Millipore, Billerica, MA), probing for the genes of interest and for the control gene (*ACTB*). Universally unmethylated DNA (Millipore, Billerica MA) was used as a negative methylation control as were several water blanks (containing no DNA template). The final composition of the master mix for QMSP consisted of 1X TaqMan® Universal PCR Master Mix No AmpErase® (Applied Biosystems, Foster City CA), 600 nM of each primer (forward and reverse) and 200 nM MGB (minor groove binder) probe (Applied Biosystems, Foster City CA), with a final reaction volume of 50 μl. Amplification conditions were as follows: 10 minutes at 95°C, followed by 95°C for 15 seconds and 60°C for 1 minute, for 50 cycles.

Bacterial Cloning and Bisulfite Sequencing

To confirm the results seen with QMSP, a small subset of samples was selected (blinded to case-control status) and bisulfite-sequenced (n=12 for *RASSF1A* and n=7 for *GSTP1*). DNA samples were sodium bisulfite treated and PCR was conducted using a final reaction volume of 25 μl. This included 1 × PCR Buffer (Qiagen, Valencia CA), 200μM dNTPs, 60nM of each (forward and reverse) primer (Applied Biosystems, Foster City CA) and 1 U Hotstart Taq Polymerase (Qiagen, Valencia CA). Conditions were 95°C for 15 minutes followed by 45 cycles of 94°C for 20 seconds, 57°C for 30 seconds and 72°C for 30 seconds, and a hold at 4°C. Product was visualized by 10% TBE polyacrylamide gel electrophoresis. Bacterial cloning reactions were carried out using the TOPO® TA Cloning Kit for sequencing (Invitrogen, Carlsbad CA) as described by the manufacturer and using One Shot TOP10F' competent cells (Invitrogen, Carlsbad CA).

Once transformed, bacteria were plated on LB (lysogeny broth) agar plates containing 0.1 mM Isopropyl β-D-1-thiogalactopyroanoside (IPTG), 0.004% galactose in dimethylfluoride and 100 μg/μl ampicillin. 100 μl of SOC (super optimal broth with catabolite repression) medium was first added to each plate, followed by 80 μl from each transformation vial (50 μl for the pUC19 transformation control). Plates were then inverted and incubated overnight at 37°C.

The next morning four colonies (1 blue and 3 white) were selected from each plate and placed in culture tubes containing 2 ml of LB medium with 100 μg/μl ampicillin. Tubes

were then incubated overnight at 37°C on a rotary shaker (225 rpm). Plasmid DNA was isolated using the PureLink Quick Plasmid Mini-prep Kit Protocol (Invitrogen, Carlsbad CA) as directed by the manufacturer. Purified plasmid DNA was stored at 4°C until use later that day or stored at −20°C if to be used the next day.

Sequencing of isolated plasmid DNA was conducted by capillary gel electrophoresis (CEQ-8000) using a Dye Terminator Cycle Sequencing (DTCS) kit (Beckman Coulter, Fullerton CA).

DNA Fragmentation Assay

To ensure that the quality of DNA obtained from the NYUWHS samples was good, fresh blood samples were collected from 6 healthy individuals by a trained phlebotomist. Serum was separated according to the protocol detailed by the NYUWHS [37] and stored at −80°C. Samples were handled in the same manner as those from the NYUWHS so that the only variable factor would be storage time. DNA was isolated using the QIAamp DNA Blood Midi Kits and stored in aliquots of 45 μl at −80°C until the time of analysis.

DNA quality was assessed using a PCR based fragment assay as described by van Beers et al [38]. For this experiment isolated DNA (10 μl) from freshly collected normal samples and study sample DNA were amplified in a multiplex PCR reaction that included 4 sets of primers specific for fragment sizes of 100, 200, 300 and 400 bps for the *GAPDH* gene. Primer sequences were obtained from van Beers et al [38] and were as follows: 100F - GTT CCA ATA TGA TTC CAC CC, 100R - CTC CTG GAA GAT GGT GAT GG, 200F – AGG TGG AGC GAG GCT AGC, 200R – TTT TGC GGT GGA AAT GTC CT, 300F – AGG TGA GAC ATT CTT GCT GG, 300R – TCC ACT AAC CAG TCA GCG TC, 400F – ACA GTC CAT GCC ATC ACT GC and 400R – GCT TGA CAA AGT GGT CGT TG.

PCR reactions for this analysis included 1 × PCR Buffer (Qiagen, Valencia CA), 200μM dNTPs, 132nM of each primer (forward and reverse) (Applied Biosystems, Foster City CA) for each fragment size and 1 U Hotstart Taq Polymerase (Qiagen, Valencia CA). Samples were then run for 15 minutes at 95°C and then 1 minute at 94°C, 1 minute at 56°C and 3 minutes at 72°C for 40 cycles followed by 7 minutes at 72°C. Reactions were then visualized on 10% TBE polyacrylamide gel electrophoresis.

Statistical Analysis

Subject characteristics were compared between the case group and each control group (controls with a history of BBD, and healthy controls) using conditional logistic regression to take into account the matched study design. To further examine the relationship between these variables and case-control status, an analysis was also conducted comparing cases to the two control groups combined as one group. In addition, a multinomial unconditional logistic regression adjusting for age was conducted to simultaneously compare all three groups.

Methylation was examined as a dichotomous variable (0 = negative/1 = positive, if there was any amplification above the threshold). The percent of fully methylated DNA (percentage of methylated reference (PMR)) was also calculated by taking the amount of DNA found to be methylated for the gene of interest, divided by the amount of *ACTB* present for that same sample, multiplied by 100. Pair-wise comparisons of the frequency of methylation were conducted using exact conditional logistic regression to take into account the low frequency of methylation and the matched design of the study. *ACTB* copy number and concentration were $\log_2$-transformed and compared between the case group and each control group also using conditional logistic regression. No additional adjustments for breast

cancer risk factors were included in the model because the objective of the study was to assess the diagnostic ability of the marker rather than assess whether it is an independent risk factor. All statistical analyses were conducted using SAS 9.1 (SAS Institute Inc., Cary NC).

Results

Nested Case-Control Study Subject Characteristics

A total of 113 cases had donated blood within the 6 months prior to diagnosis. However, due to the precious nature of these prospectively collected samples, it was decided a priori that DNA methylation analysis would be completed in two stages, with the first stage limited to the 50 cases who had the highest number of serum aliquots in storage. Because the results in these first 50 cases and their matched controls did not support our initial hypothesis, methylation analysis was not extended to the remaining cases.

Table 1 shows that cases and controls were comparable. No significant differences were seen for any variable between any case-control group, thus for simplicity, the odds ratios are not presented. The median age at index date (the date of diagnosis for cases and the corresponding date for their matched controls) was 52.0 years for cases, 51.5 years for BBD controls and 51.8 years for healthy controls. The majority of women were postmenopausal at baseline, and these proportions were similar in all three subject groups (56% of cases, 57% of healthy controls and 58% of BBD controls). The median number of days between the date of blood donation and the date of diagnosis was 40.5 days with a range of 5 – 175 days. Sixty-eight percent of cases were invasive ductal carcinomas, 75% were estrogen receptor-positive and 47% of cases were diagnosed with stage I disease (Table 1).

Methylation Analysis (QMSP)

ACTB copy number was used to determine the amount of DNA in each sample. The median *ACTB* copy number was 6,375 copies/ml of serum for healthy controls, 5,404 copies/ml for BBD controls and 5,978 copies/ml for cases (Table 2). This corresponds to an overall median (in all subjects) of approximately 20ng of DNA per ml of serum. No statistical differences in *ACTB* amount were observed between cases and either control group. The *ACTB* copy number was higher in subjects who were later diagnosed with stage II tumors (median = 7,201 copies/ml; 10^{th}-90^{th} percentiles, 251- 18,551) compared to those with stage I tumors (median = 4,242 copies/ml, 10^{th}-90^{th} percentiles, 1,068-16,683), although this difference was not statistically significant (p=0.98, Student t-test of $\log_2$ transformed *ACTB* copy number/ml).

Table 3 shows the frequency (95% confidence interval (CI)) of methylation in cases and controls, as determined by amplification above the threshold (PMR>0). The methylation frequencies of the four genes analyzed were low among all three subject groups. Further, these frequencies did not differ between cases, controls with a history of BBD and controls without a history of BBD.

Because no significant differences between the two control groups were observed, the control groups were combined and the analysis repeated. Again, no significant differences in the frequency of methylation were observed between cases and controls. Only for *RASSF1A* and *RARβ2* was the frequency higher (non-significantly) in cases than controls (*RASSF1A*: 22% of cases, 19% of controls; *RARβ2*: 6.7% of cases and 1.5% or controls). Among those women with a PMR>0, PMR values did not differ between cases and the combined control group for any gene (results not shown), though the interpretation of these comparisons is limited by the small number of subjects with a PMR>0 (n=39 for *RASSF1A*, n=14 for

GSTP1, n=5 for *RARβ2* and n=7 for *APC*). Overall, 31.8% of cases and 28.8% of controls (BBD and healthy controls combined) had methylation in at least one gene.

The methylation assays were shown to have good sensitivity using a series of standards (dilutions of fully methylated DNA), able to detect down to one genome copy. Standards were shown to have a high level of reproducibility between plates, as indicated by low inter-plate coefficients of variability (CV) for each gene: *RASSF1A*: 11%; *GSTP1*: 3%; *APC*: 2%; *RARβ2*: 1%. Because *ACTB* was the only gene for which multiple measurements were available for each study subject on different plates the reproducibility of these repeat measures were examined. As was seen with the standards included in each plate, the inter-plate CV for Ct values (the Ct value is the point at which amplification crosses the detection threshold) for *ACTB* was 3%. The CV did not differ between cases and either control group. Though the inter-plate CV for the Ct values was low, the CV for the corresponding copy number was 53.2%. This increase in variability is seen because a small difference in Ct translates into a large difference in copy number once the copy number is log transformed and made linear.

Bisulfite Sequencing

Twelve samples that amplified for *RASSF1A* using QMSP were re-analyzed using bisulfite sequencing. Of these, ten were found to have methylated *RASSF1A* promoters by the sequencing method. Of the seven samples that amplified for *GSTP1* using QMSP that were re-analyzed using bisulfate sequencing, none were found to be methylated.

Fragmentation Analysis

Results of the fragment analysis showed that, though the fresh samples and the NYUWHS samples looked somewhat different (i.e. the NYUWHS samples had a greater amount of DNA), the NYUWHS samples were not more fragmented than the fresh DNA samples (Figure 1). Analysis was repeated with the same outcome.

Discussion

We conducted a nested case-control study to examine the potential of promoter methylation of four cancer-related genes (*RASSF1A*, *GSTP1 RARβ2* and *APC*) in serum DNA as a marker for the early detection of breast cancer. Cases were women who donated blood in the 6 months preceding their diagnosis of breast cancer. We found that promoter methylation of these four genes, as determined by a PMR > 0 (i.e. any amplification beyond the threshold) [24], was unable to distinguish between cases and controls. We therefore discontinued our study after analysis of the first 50 cases and their matched controls, so as not to waste valuable prospectively collected samples.

Most previous studies of methylation in serum/plasma DNA used samples collected at or after breast cancer diagnosis [22-30]. Prospective cohort studies, such as the NYUWHS, are needed to assess the ability of promoter methylation to detect pre-clinical disease [39-40]. While one study showed that *RASSF1A* methylation (assessed by methylation-specific PCR) was detectable in plasma as early as 7 years prior to breast cancer diagnosis [41], our study is, to our knowledge, the first to assess promoter methylation of several genes in serum samples collected prior to diagnosis of breast cancer.

The serum samples used in this study were collected in 1985-1991 and thus had been stored for approximately 20 years at −80°C at the time the assays were performed. We considered whether the long-term storage could have affected the methylation results. A high proportion of smaller DNA fragments (high degree of DNA fragmentation) can disrupt the detection of promoter methylation if it occurs at the site of primer/probe binding. One measure used to

avoid this possibility in this study was to use QMSP primers that have been designed to create a PCR product that is usually less than 100bp. Still, if the NYUWHS samples have a higher degree of fragmentation, this could lead to the generation of false-negative results and a lower than expected frequency of methylation. The fragment analysis however indicated that the NYUWHS samples were not more fragmented than the fresh DNA samples, suggesting that the quality of the DNA in the NYUWHS samples was good and not responsible for the results observed.

Serum/plasma methylation frequencies observed in breast cancer cases in previous studies were 23-80% for *RASSF1A*, 26% for *GSTP1*, 0-26% for *RAR•2* and 17-80% for *APC* (reviewed in [42]), compared to 22.0% for *RASSF1A*, 4.0% for *GSTP1*, 6.7% for *RAR•2* and 2.0% for *APC* in our study. The lower frequencies seen in cases in our study as compared to other studies could be due to false negative results (i.e. truly methylated samples classified as non-methylated). False negative results could be attributed to the low amount of DNA template used in the assay. The absolute sensitivity of an assay is the minimum quantity of target DNA required for successful amplification and detection [43]. Under ideal conditions the sensitivity of QMSP has been estimated to be 1 methylated copy within 10,000 copies of unmethylated normal DNA [34]. This suggests that a minimum of 10,000 copies of DNA template in each PCR reaction (in practice 30,000 copies) is required to achieve optimal sensitivity. This corresponds to 99 ng of DNA per PCR reaction (30,000 genome copies × 3.3 pg [(the weight of one haploid genome copy]). In our study, one milliliter of serum yielded a median amount of DNA of 5,978 *ACTB* copies, corresponding to approximately 20 ng/ml of DNA (the actual amount of circulating DNA in these subjects is likely more than this since some will be lost during the bisulfite treatment procedure). Though this is in the range of DNA concentrations (10-1000 ng/ml) found in serum in other studies [26,43-45], it is about 5 times less than the amount hypothetically required to achieve optimal sensitivity, supporting the potential for false-negative results in our study.

Studies have typically included only small numbers of often convenience based controls and reported methylation frequencies of 0-10% for *RASSF1A*, 0% for *GSTP1*, 8% for *RAR•2* and 0% for *APC* (reviewed in [42]). Methylation frequencies observed in the controls in our study were 17.2% for *RASSF1A*, 7.1% for *GSTP1*, 4.2% for *APC* and 1.1% for *RAR•2* in healthy controls and 22.9% for *RASSF1A*, 10.4% for *GSTP1*, 4.4 for *APC* and 2.3 for *RAR•2* in controls with a history of BBD. The somewhat higher frequencies of methylation observed in controls in our study, as compared to other studies, suggested the possibility that some samples were erroneously classified as methylated (false positives). This could be due to non-specific amplification after a high number of PCR cycles (i.e. QMSP was run for 50 cycles). Our re-analyses of some samples by bisulfite sequencing, the gold standard for methylation analysis, confirmed this hypothesis. For *RASSF1A*, the percent of samples misclassified as methylated was 17% (2 out of 12) and for *GSTP1*, it was 100% (7 out of 7). These results confirmed the potential for non-specific amplification after a high number of cycles (generating false-positives), though the rate of occurrence appears to be variable depending on the gene being investigated.

We used QMSP, rather than bisulfite sequencing, as the primary method of analysis because bisulfite sequencing is labor-intensive and expensive and also because of the limited amount of biological material available. In addition to the indication of the presence of both false-negative and false-positive results, a lack of reproducibility in repeat *ACTB* measurements was observed with this method. Few studies have documented the reproducibility of methylation measurements by QMSP. One study designed to specifically examine the reproducibility of the PMR, was based on QMSP analysis of DNA from paraffin-embedded colon cancer samples. This study found the PMR to have high inter-assay CVs with an average of 21% (range 10-38%) [46]. To our knowledge, this study is the first to report CVs

for repeat QMSP analysis using samples containing small amounts of DNA template obtained from serum. The high variability seen in these measurements indicate that QMSP may not be the ideal method for methylation analysis when there is only a small amount of DNA template available (e.g., analysis of prediagnostic serum samples).

Because studies have generally included only small numbers of controls 'normal' patterns of DNA methylation are poorly characterized. Studies of high-risk asymptomatic women have found relatively frequent methylation of some tumor suppressor and other cancer-related genes [47-48]. For example, a study including fine-needle aspiration biopsies from 55 healthy women at a high risk of developing breast cancer (as determined by the Gail model), detected promoter methylation of *RARβ2* (9%), *APC* (26%), *H-cadherin* (17%) and *RASSF1A* (37%) [48]. Little data, though, are available for average-risk individuals. Age related changes in methylation have been shown and a strong environmental component identified [49-50]. A prospective design, such as the one used, may contribute knowledge on the frequency of methylation in "healthy" individuals, in addition to ensuring that cases and controls are comparable with respect to demographic and socio-economic characteristics and that controls do not have subclinical cancer. The estimates obtained from our study should be interpreted with caution, due to the possibility for false-positive results discussed above.

Finally, the genes included in this study were selected based on their known role in carcinogenesis and because they have previously been shown to be methylated in a substantial proportion of breast cancers [13-15,18,31]. However, only four genes were included and it is possible that other genes might have led to better differentiation between cases and controls. The inclusion of a larger number of genes in future studies would be helpful. However, each additional gene will increase the amount of DNA required, underscoring the need for assay methods applicable to small amounts of DNA.

Promoter methylation in circulating DNA remains a candidate marker for early detection of breast cancer. As outlined by Pepe et al [39], one of the steps in the evaluation of biomarkers for early diagnosis of disease is to determine the optimal method for measuring the biomarker and to characterize those factors, environmental, lifestyle and genetic that are associated with biomarker status. The results of the current study show that QMSP may not be the optimal method of methylation analysis of prospectively collected samples, where only small amounts of biological material are available. Validation of methods of analysis for the very small amounts of DNA usually available in prospective studies is needed, as well as further characterization of the methylation profile in the serum DNA of healthy individuals.

Acknowledgments

The authors thank the participants of the New York University Women's Health Study (NYUWHS) whose participation made this project possible. This work was primarily funded through the research grant BCTR0707521 from Susan G. Komen for the Cure. The NYUWHS is supported by research grants R01CA034588, R01CA098661, center grant P30CA016087 from the National Cancer Institute and the center grant ES000260 from the National Institute of Environmental Health Science.

Grant Support: JDB: DOD Pre-doctoral Training Grant BC060891. JDB, AZJ, CBK: Susan G. Komen for the Cure BCTR0707521, R01 CA098661, AZJ: National Cancer Institute Cancer Center Grant CA-016087, and the National Institute of Environmental Health Sciences center grant ES-000260. PC: NIH Early Detection Research Network 1 U01 CA111242.

References

1. Etzioni R, Urban N, Ramsey S, McIntosh M, Schwartz S, Reid B, et al. The case for early detection. Nat Rev Cancer. 2003; 3(4):243–252. [PubMed: 12671663]

2. Weir HK, Thun MJ, Hankey BF, Ries LAG, Howe HL, Wingo PA, et al. Annual Report to the Nation on the Status of Cancer, 1975-2000, Featuring the Uses of Surveillance Data for Cancer Prevention and Control. J. Natl. Cancer Inst. 2003; 95(17):1276–1299. [PubMed: 12953083]

3. Cancer Trends Progress Report - 2007 Update. National Cancer Institute, NIH, DHHS; Bethesda, MD: [December 2007]. http://progresspreport.cancer.gov

4. Berg WA, Gutierrez L, NessAiver MS, Carter WB, Bhargavan M, Lewis RS, et al. Diagnostic Accuracy of Mammography, Clinical Examination, US, and MR Imaging in Preoperative Assessment of Breast Cancer. Radiology. 2004; 233(3):830–849. [PubMed: 15486214]

5. Evans A. Hormone Replacement Therapy and Mammographic Screening. Clinical Radiology. 2002; 57(7):563–564. [PubMed: 12096852]

6. Zhi H, Ou B, Luo B-M, Feng X, Wen Y-L, Yang H-Y. Comparison of Ultrasound Elastography, Mammography, and Sonography in the Diagnosis of Solid Breast Lesions. J Ultrasound Med. 2007; 26(6):807–815. [PubMed: 17526612]

7. Maskarinec G, Meng L, Ursin G. Ethnic differences in mammographic densities. Int. J. Epidemiol. 2001; 30(5):959–965. [PubMed: 11689504]

8. Levenson VV. Biomarkers for early detection of breast cancer: What, when, and where? Biochimica et Biophysica Acta (BBA) - General Subjects. 2007; 1770(6):847–856.

9. Lopez JK, Bassett LW. Invasive Lobular Carcinoma of the Breast: Spectrum of Mammographic, US, and MR Imaging Findings. Radiographics. 2009; 29(1):165–176. [PubMed: 19168843]

10. Elmore JG, Barton MB, Moceri VM, Polk S, Arena PJ, Fletcher SW. Ten-Year Risk of False Positive Screening Mammograms and Clinical Breast Examinations. N Engl J Med. 1998; 338(16):1089–1096. [PubMed: 9545356]

11. Ernster V, Barclay J. Increases in ductal carcinoma in situ (DCIS) of the breast in relationto mammography: a dilemma. J Natl Cancer Inst Monogr. 1997; 22:151–156. [PubMed: 9709292]

12. Jones PA, Baylin SB. The Epigenomics of Cancer. Cell. 2007; 128(4):683–692. [PubMed: 17320506]

13. Fackler M, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak K, et al. DNA methylation of RASSF1A, HIN-1, RAR-β, Cyclin D2 and TWIST in in situ and invasive lobular breast carcinoma. International Journal of Cancer. 2003; 107(6):970–975.

14. Fackler MJ, McVeigh M, Mehrotra J, Blum MA, Lange J, Lapides A, et al. Quantitative Multiplex Methylation-Specific PCR Assay for the Detection of Promoter Hypermethylation in Multiple Genes in Breast Cancer. Cancer Res. 2004; 64(13):4442–4452. [PubMed: 15231653]

15. Parrella P, Poeta ML, Gallo AP, Prencipe M, Scintu M, Apicella A, et al. Nonrandom Distribution of Aberrant Promoter Methylation of Cancer-Related Genes in Sporadic Breast Tumors. Clin Cancer Res. 2004; 10(16):5349–5354. [PubMed: 15328171]

16. Jeronimo C, Costa I, Martins MC, Monteiro P, Lisboa S, Palmeira C, et al. Detection of Gene Promoter Hypermethylation in Fine Needle Washings from Breast Lesions. Clin Cancer Res. 2003; 9(9):3413–3417. [PubMed: 12960130]

17. Tao M, Shields P, Nie J, Millen A, Ambrosone C, Edge S, et al. DNA hypermethylation and clinicopathological features in breast cancer: the Western New York Exposures and Breast Cancer (WEB) Study. Breast Cancer Research and Treatment. 2009; 114(3):559–568. [PubMed: 18463976]

18. Shinozaki M, Hoon DSB, Giuliano AE, Hansen NM, Wang H-J, Turner R, et al. Distinct Hypermethylation Profile of Primary Breast Cancer Is Associated with Sentinel Lymph Node Metastasis. Clin Cancer Res. 2005; 11(6):2156–2162. [PubMed: 15788661]

19. Li S, Rong M, Iacopetta B. DNA hypermethylation in breast cancer and its association with clinicopathological features. Cancer Letters. 2006; 237(2):272–280. [PubMed: 16029926]

20. Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102(45):16368–16373. [PubMed: 16258065]

21. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch R-D, et al. DNA Fragments in the Blood Plasma of Cancer Patients: Quantitations and Evidence for Their Origin from Apoptotic and Necrotic Cells. Cancer Res. 2001; 61(4):1659–1665. [PubMed: 11245480]

22. Hoque MO, Feng Q, Toure P, Dem A, Critchlow CW, Hawes SE, et al. Detection of Aberrant Methylation of Four Genes in Plasma DNA for the Detection of Breast Cancer. J Clin Oncol. 2006; 24(26):4262–4269. [PubMed: 16908936]

23. Müller HM, Widschwendter A, Fiegl H, Ivarsson L, Goebel G, Perkmann E, et al. DNA Methylation in Serum of Breast Cancer Patients: An Independent Prognostic Marker. Cancer Res. 2003; 63(22):7641–7645. [PubMed: 14633683]

24. Papadopoulou E, Davilas E, Sotiriou V, Georgakopoulos E, Georgakopoulou S, Koliopanos A, et al. Cell-free DNA and RNA in Plasma as a New Molecular Marker for Prostate and Breast Cancer. Ann NY Acad Sci. 2006; 1075(1):235–243. [PubMed: 17108217]

25. Tan S-H, Ida H, Lau Q-C, Goh B-C, Chieng W-S, Loh M, et al. Detection of promoter hypermethylation in serum samples of cancer patients by methylation-specific polymerase chain reaction for tumour suppressor genes including RUNX3. Oncol Rep. 2007; 18(5):1225–1230. [PubMed: 17914577]

26. Dulaimi E, Hillinck J, de Caceres II, Al-Saleem T, Cairns P. Tumor Suppressor Gene Promoter Hypermethylation in Serum of Breast Cancer Patients. Clin Cancer Res. 2004; 10(18):6189–6193. [PubMed: 15448006]

27. Sharma G, Mirza S, Prasad CP, Srivastava A, Gupta SD, Ralhan R. Promoter hypermethylation of p16INK4A, p14ARF, CyclinD2 and Slit2 in serum and tumor DNA from breast cancer patients. Life Sciences. 2007; 80(20):1873–1881. [PubMed: 17383681]

28. Mirza S, Sharma G, Prasad CP, Parshad R, Srivastava A, Gupta SD, et al. Promoter hypermethylation of TMS1, BRCA1, ER[alpha] and PRB in serum and tumor DNA of invasive ductal breast carcinoma patients. Life Sciences. 2007; 81(4):280–287. [PubMed: 17599361]

29. Shukla S, Mirza S, Sharma G, Parshad R, Gupta SD, Ralhan R. Detection of RASSF1A and RARbeta Hypermethylation in Serum DNA from Breast Cancer Patients. Epigenetics. 2006; 1(2): 88–93. [PubMed: 17998817]

30. Hu X-C, Wong I, Chow L. Tumor-derived aberrant methylation in plasma of invasive ductal breast cancer patients: Clinical implications. Oncology Rep. 2003; 10:1811–1815.

31. Jeronimo C, Monteiro P, Henrique R, Dinis-Ribeiro M, Costa I, Costa V, et al. Quantitative hypermethylation of a small panel of genes augments the diagnostic accuracy in fine-needle aspirate washings of breast lesions. Breast Cancer Research and Treatment. 2008; 109(1):27–34. [PubMed: 17549626]

32. Zeleniuch-Jacquotte A, Shore R, Koenig K, Akhmedkhanov A, Afanasyeva Y, Kato I, et al. Postmenopausal levels of oestrogen, andorgen, and SHBG and breast cancer: long-term results of a prospective study. British Journal of Cancer. 2004; 90:153–159. [PubMed: 14710223]

33. Kato I, Toniolo P, Koenig KL, Kahn A, Schymura M, Zeleniuch-Jacquotte A. Comparison of active and cancer registry-based follow-up for breast cancer in a prospective cohort study. Am J Epidemiol. 1999; 149:372–378. [PubMed: 10025481]

34. Eads CA, Lord RV, Kurumboor SK, Wickramasinghe K, Skinner ML, Long TI, et al. Fields of Aberrant CpG Island Hypermethylation in Barrett's Esophagus and Associated Adenocarcinoma. Cancer Res. 2000; 60(18):5021–5026. [PubMed: 11016622]

35. Widschwendter M, Siegmund KD, Muller HM, Fiegl H, Marth C, Muller-Holzner E, et al. Association of Breast Cancer DNA Methylation Profiles with Hormone Receptor Status and Response to Tamoxifen. Cancer Res. 2004; 64(11):3807–3813. [PubMed: 15172987]

36. Schmiemann V, Bocking A, Kazimirek M, Onofre ASC, Gabbert HE, Kappes R, et al. Methylation Assay for the Diagnosis of Lung Cancer on Bronchial Aspirates: A Cohort Study. Clin Cancer Res. 2005; 11(21):7728–7734. [PubMed: 16278393]

37. Toniolo P, Levitz M, Zeleniuch-Jacquotte A, Banerjee S, Koenig K, Shore R, et al. A prospective study of endogenous estrogens and breast cancer in postmenopausal women. J Natl Cancer Inst. 1995; 87(3):190–197. [PubMed: 7707406]

38. van Beers E, Joosse S, Lightenberg M, Fles R, Hogervorst F, Verhoef S, et al. A Multiplex PCR Predictor for aCGH success of FFPE samples. British Journal of Cancer. 2006; 94:333–337. [PubMed: 16333309]

39. Pepe MS, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, et al. Phases of Biomarker Development for Early Detection of Cancer. J Natl Cancer Inst. 2001; 93(14):1054–1061. [PubMed: 11459866]
40. Pepe MS. Evaluating technologies for classification and prediction in medicine. Statistics in Medicine. 2005; 24(24):3687–3696. [PubMed: 16320261]
41. Yazici H, Terry MB, Cho YH, Senie RT, Liao Y, Andrulis I, et al. Aberrant Methylation of RASSF1A in Plasma DNA Before Breast Cancer Diagnosis in the Breast Cancer Family Registry. Cancer Epidemiol Biomarkers Prev. 2009; 18(10):OF1–3.
42. Brooks J, Cairns P, Zeleniuch-Jacquotte A. Promoter methylation and the detection of breast cancer. Cancer Causes and Control. 2009
43. Cottrell SE, Laird PW. Sensitive Detection of DNA Methylation. Ann NY Acad Sci. 2003; 983(1): 120–130. [PubMed: 12724217]
44. Wu T-L, Zhang D, Chia J-H, Tsao K-C, Sun C-F, Wu JT. Cell-free DNA: measurement in various carcinomas and establishment of normal reference range. Clinica Chimica Acta. 2002; 321(1-2): 77–87.
45. Taback B, O'Day SJ, Hoon DSB. Quantification of Circulating DNA in the Plasma and Serum of Cancer Patients. Annals of the New York Academy of Sciences. 2004; 1022:17–24. (CIRCULATING NUCLEIC ACIDS IN PLASMA/SERUM III AND SERUM PROTEOMICS). [PubMed: 15251934]
46. Ogino S, Kawasaki T, Brahmandam M, Cantor M, Kirkner GJ, Spiegelman D, et al. Precision and Performance Characteristics of Bisulfite Conversion and Real-Time PCR (MethyLight) for Quantitative DNA Methylation Analysis. J Mol Diagn. 2006; 8(2):209–217. [PubMed: 16645207]
47. Vasilatos SN, Broadwater G, Barry WT, Baker JC Jr. Lem S, Dietze EC, et al. CpG Island Tumor Suppressor Promoter Methylation in Non-BRCA-Associated Early Mammary Carcinogenesis. Cancer Epidemiol Biomarkers Prev. 2009; 18(3):901–914. [PubMed: 19258476]
48. Lewis CM, Cler LR, Bu D-W, Zochbauer-Muller S, Milchgrub S, Naftalis EZ, et al. Promoter Hypermethylation in Benign Breast Epithelium in Relation to Predicted Breast Cancer Risk. Clin Cancer Res. 2005; 11(1):166–172. [PubMed: 15671542]
49. Fraga M, Agrelo R, Esteller M. Cross-Talk between Aging and Cancer. Annals of the New York Academy of Sciences. 2007; 1100:60–74. (Biogerontology Mechanisms and Interventions). [PubMed: 17460165]
50. Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, et al. From The Cover: Epigenetic differences arise during the lifetime of monozygotic twins. PNAS. 2005; 102(30): 10604–10609. [PubMed: 16009939]

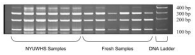

Figure 1. Fragment Analysis of Freshly Collected and NYUWHS DNA[a]

[a]Results of the fragment analysis for GAPDH in NYUWHS and freshly collected samples as described by van Beers et al [38]. The fragment analysis indicated that the NYUWHS samples were not more fragmented than the fresh DNA samples, suggesting that the quality of the DNA in the NYUWHS samples was good and not responsible for the results observed.

Table 1

Subject Characteristics[a]

Variable	Cases (n=50)	BBD Control (n=50)	Healthy Control (n=100)
Age at Index Date (years)			
Median (10^{th}, 90^{th} percentile)	52.0 (40.5, 65.9)	51.5 (40.4, 66.0)	51.8 (40.4, 65.8)
Menopausal Status, n (%)			
Premenopausal	22 (44)	21 (42)	43 (43)
Postmenopausal	28 (56)	29 (58)	57 (57)
BMI (kg/m^2)			
Median (10^{th}, 90^{th} percentile)			
≤52 years	23.4 (20.4, 29.0)	21.8 (19.1, 27.3)	25.5 (19.8, 32.8)
>52 years	25.8 (22.7, 31.0)	23.2 (21.4, 28.3)	24.4 (21.5, 31.2)
Height (cm)			
Median (10^{th}, 90^{th} percentile)	162.6 (154.9, 170.2)	162.6 (152.4, 170.2)	162.6 (154.9, 172.7)
Ethnicity, n (%)			
Caucasian	37 (74.0)	37 (80.4)	74 (78.7)
Black	8 (16.0)	4 (8.7)	13 (13.8)
Other (incl. Hispanic and Asian)	5 (10.0)	5 (10.9)	7 (7.5)
Unknown	0	4	6
Family History, n (%)			
None	42 (84.0)	40 (80.0)	77 (77.0)
1 affected relative, >45 yrs	4 (8.0)	10 (20.0)	15 (15.0)
>1 affected relative, or 1 age <45 yrs	4 (8.0)	0 (0.0)	8 (8.0)
Age at First Term Pregnancy, n (%)			
<25	16 (32.0)	24 (48.0)	34 (34.0)
25-29	12 (24.0)	6 (12.0)	22 (22.0)
Nulliparous	12 (24.0)	15 (30.0)	33 (33.0)
30+	10 (20.0)	5 (10.0)	11 (11.0)
Oophorectomy, n (%)			
Yes	1 (2.0)	3 (6.0)	9 (9.1)
Smoking, n (%)			
Never	19 (45.2)	25 (53.2)	49 (51.6)
Current	8 (19.1)	9 (19.2)	19 (20.0)
Past	15 (35.7)	13 (27.6)	27 (28.4)
Unknown	8	3	5
OC Use, n (%)			
Ever	16 (43.2)	19 (38.8)	37 (41.1)
HRT Use, n (%)			
Ever	4 (8.0)	8 (16.0)	8 (8.0)
Histology			
Invasive ductal carcinoma	34 (68.0)		

Variable	Cases (n=50)	BBD Control (n=50)	Healthy Control (n=100)
Invasive lobular carcinoma	6 (12.0)		
Invasive mucinous carcinoma	1 (2.0)		
Invasive medullary carcinoma	3 (6.0)		
Paget's disease of the breast/nipple	1 (2.0)		
Non-specified invasive histology	5 (10.0)		
Stage			
1	20 (47.6)		
2A	17 (40.5)		
2B	3 (7.1)		
3A	1 (2.4)		
Unknown	8		
ER Status			
Postivie	24 (75.0)		
Negative	8 (25.0)		
Unknown	18		
PR Status			
Postivie	19 (66.0)		
Negative	10 (34.0)		
Unknown	21		

[a] No statistical differences between any case-control groups were observed (conditional logistic regression).

Table 2

Median Copy Number and Concentration of *ACTB* by Case-Control Status*

	Median ACTB Copy Number/ml (10^{th}, 90^{th} percentile)	Median ACTB (ng/ml) (10^{th}, 90^{th} percentile)
Healthy Controls	6,375 (1,075 - 28,751)	21.0 (3.6 - 94.9)
BBD Controls	5,404 (268 - 45,002)	17.8 (0.9 - 148.5)
Cases	5,978 (444 - 23,823)	19.7 (1.5 - 78.6)

* No significant differences were observed between any case-control group (conditional logistic regression).

Table 3

Frequency of Promoter Methylation by Case-Control Status[A, B]

	RASSF1A	GSTP1	APC	RARβ2	At least One Gene Methylated[C]
	Frequency % (n) 95% CI	**Frequency % (n) 95% CI**	**Frequency % (n) 95% CI**	**Frequency % (n) 95% CI**	**Frequency % (n) 95% CI**
Cases	22.0 (11/50)	4.0 (2/50)	2.0 (1/49)	6.7 (3/45)	31.8 (14/44)
	10.5, 33.5	−1.4, 9.4	−1.9, 5.9	−0.6, 14.0	18.0, 45.6
BBD	22.9 (11/48)	10.4 (5/48)	4.4 (2/46)	2.3 (1/43)	38.4 (15/39)
Controls	11.0, 34.8	1.8, 19.0	−1.5, 10.3	−2.2, 6.8	24.5, 52.3
Healthy	17.2 (17/99)	7.1 (7/99)	4.2 (4/96)	1.1 (1/88)	24.4 (21/86)
Controls	9.8, 24.6	2.0, 12.2	0.2, 8.2	−1.1, 3.3	15.3, 33.5

[A] Methylation Frequencies (PMR>0) by case-control status, no statistical differences were found (Conditional logistic regression).

[B] 7 subjects were excluded due to undetectable ACTB levels in at least one of the assays (4 BBD controls and 3 healthy controls). In the case of RARβ2 additional samples did not amplify in the first round of analysis and were not repeated due to the precious nature of the samples and the results of the completed genes indicating that the frequency of methylation did not differ between cases and controls.

[C] Includes those women who had methylation measurements for all four genes