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Cancer Causes Control. Author manuscript; available in PMC 2010 November 1.

Published in final edited form as:

Cancer Causes Control. 2009 November ; 20(9): 1539–1550. doi:10.1007/s10552-009-9415-y.

Promoter Methylation and the Detection of Breast Cancer

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Abstract

Mammographic screening has been shown to reduce breast cancer mortality in women over the age of 50 years, and to a lesser extent in younger women. The sensitivity of mammography however, is reduced in some groups of women. There remains a need for a minimally invasive, cost-effective procedure that could be used along side mammography to improve screening sensitivity.

Silencing of tumor suppressor genes through promoter hypermethylation is known to be a frequent and early event in carcinogenesis. Further, changes in methylation patterns observed in tumors are also detectable in the circulation of women with breast cancer. This makes these alterations candidate markers for early tumor detection.

In this paper we review the current literature on promoter hypermethylation changes and breast cancer and discuss issues that remain to be addressed in order for the potential of these markers to augment the sensitivity of screening mammography. In general, studies in well defined populations, including appropriate controls and larger numbers are needed. Further, focus on the optimization of methods of methylation detection in small amounts of DNA is needed.

Keywords

Breast Neoplasms; Early Detection of Cancer; DNA Methylation

In the United States, over 12% of women born today can expect to develop breast cancer in their lifetime (1). Women who are diagnosed at an early stage of disease have a better prognosis and require less severe treatment regimens than those diagnosed at an advanced stage (2). Regular mammograms have been found to reduce breast cancer mortality in women over 50 years old and to a lesser extent in younger women (3-5), leading to the current recommendations that women at average risk should receive mammograms every 1 or 2 years, beginning at age forty (6). Indeed, in the US, the majority of women over the age of 40 undergo mammographic screening (6,7). Suspicious mammographic findings lead to further testing that may include other imaging techniques such as magnetic resonance (MRI) and/or ultrasound, but ultimately the diagnosis is established by a biopsy, in particular to differentiate malignant from benign tumors. Although this well established screening approach has led to a reduction in breast cancer mortality, it has a number of limitations pointing to the need for additional, complementary modalities which we briefly review below.

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Need for Complementary Breast Screening Modalities

The sensitivity of screening mammography varies from about 68 to 93% (8), due to variations in practitioners' experience and skill (9) and patient characteristics. Specifically, mammography is up to 50% less sensitive in women who are young, Asian, on HRT, and/or have dense breasts (10-13). It is also less sensitive for the detection of invasive lobular carcinomas (ILC) and small or diffuse tumors (9,14,15).

Specificity is an issue as well, 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 (16,17). Another concern is that a lifetime of yearly mammographic screening may eventually lead to a level of radiation exposure that increases breast cancer risk (18).

In light of these limitations, a minimally invasive screening test administered at the time of mammography, or prior to biopsy in the case of a suspicious mammogram, that would lead to overall greater sensitivity and specificity could have important public health value. To be maximally effective, this procedure should be able to provide information where mammography has deficiencies. That is, it should have the ability to distinguish between benign and malignant tumors and improve the sensitivity of detection for lobular carcinomas. It should also improve the sensitivity of detection in women with high breast density.

DNA Methylation in Cancer

Cancer initiation and progression is driven by the accumulation of inherited or acquired DNA mutations. These alterations may be genetic or epigenetic in nature (19). Epigenetic modifications are changes in DNA structure that do not involve sequence changes but are stably inherited from cell to cell. These include DNA methylation, histone modifications (phosphorylation, acetylation, methylation) and microRNAs. Though these modifications may all show potential for early detection of cancer, this review focuses exclusively on DNA methylation.

Methylation of cytosine located 5' to a guanosine can occur across the genome, but most notably within 0.5-4kb CpG dinucleotide rich regions, known as CpG islands (20-22). Under normal conditions, the vast majority of CpG sites in the genome are methylated, with the exception of CpG islands located 5' to the promoter and exon 1 of more than 50% of genes (22). Methylation of gene promoter CpG islands is tightly linked to histone modifications and chromosome remodeling mechanisms that lead to gene silencing (23). This method of controlling gene expression is widely used throughout the healthy genome. It is involved in the regulation of tissue- and time-specific gene expression (during differentiation and development), X chromosome inactivation in women, establishment and maintenance of imprinted genes and the silencing of transposable elements (20).

The disruption of normal methylation patterns has been found to be an important event in carcinogenesis. In general, a shift to local promoter CpG island hypermethylation is seen within the context of an overall loss of methylation (hypomethylation). While global hypomethylation is thought to play a role in carcinogenesis primarily by increasing genetic instability, local hypermethylation alters gene expression (19). Silencing of tumor suppressor genes through promoter hypermethylation is known to be a common event in carcinogenesis, thought to provide a selective growth advantage to tumor cells and contributing to the overall genetic instability of the tumor. This hypermethylation appears to be an early event in carcinogenesis (21,24,25), and occurs at least as frequently as genetic mutations in somatic cells so that hundreds of genes may be inactivated by DNA methylation in a single cancer (23,26). A large number of studies of breast cancer tissue have been conducted showing the frequent methylation of genes involved in cell cycle regulation: *p16*^{INK4A}, *p14*^{ARF}, *p15, CCDN2,*

DAPK; DNA repair: *MGMT, hMLH1*; transformation: *GSTP1*; signal transduction: *RARβ2, APC, ERβ*; and adhesion and metastasis: *CDH1, CDH13*. The high frequency with which these alterations occur in cancer makes them potentially useful markers of disease.

Methylation Markers in Circulating DNA

Mandel and Metais first discovered cell-free nucleic acids in the general circulation in the late 1940's (27). DNA is released into the circulation in healthy individuals and to a greater extent during pregnancy (from the placenta), trauma and after organ transplantation (28). DNA is also present in the circulation of people with cancer (29) and in these individuals it has been estimated that as much as 93% of the total circulating DNA is derived from the tumor (30). The mechanism of DNA release into circulation is poorly understood, but it is believed that DNA is released during tumor necrosis and apoptosis (30). Because circulating levels of DNA are highly variable and are not limited to individuals with cancer, DNA concentration alone makes a poor cancer diagnostic tool, it can however, be a source of biomarkers.

It was first recognized that tumors were the origin of some circulating DNA during the mid-nineties when it was found that it was possible to detect cancer-associated mutations (*N-ras, k-ras*) (28), micro-satellite instability and loss of heterozygosity (LOH) identical to that seen in the tumor (31). DNA alterations, including both mutations and epigenetic modifications, have also been detected in patients with small and *in situ* lesions. This suggests that tumors are releasing DNA early in the disease process, even before they become invasive (23) and therefore that circulating DNA may be a source of markers for tumor detection.

A number of studies illustrate the potential for the use of methylation markers in the early detection of a variety of cancers including prostate (32,33), bladder (34,35), gastric (36), renal (37), ovarian (38), colorectal (39), cervical (40), lung (41-43), liver (44) and breast (32-34, 37,38,45). Studies in lung cancer have found that aberrant DNA methylation is detectable as early as 3 years prior to diagnosis in the sputum of subjects exposed to carcinogens (uranium miners and smokers) (42). In a study of hepatocellular carcinoma, Santella et al (44) detected changes in serum methylation patterns of *RASSF1A, p16* and *p15* (using DNA from 200μl of serum) as much as 9 years prior to diagnosis.

DNA Methylation and Breast Cancer – Results From Tumor Tissue

Table 1 shows that some genes are frequently methylated in tumor tissue DNA obtained from women who have been diagnosed with breast cancer (24,46-51). These studies have been conducted using a wide range of gene panels, though there is overlap among them. The sensitivity of detection, which ranges from 60 to 100% depends on the gene panel and the histological type of breast cancer. Studies have also shown that aberrant methylation events occur early on in breast cancer development, and are detectable in tissue from *in situ* carcinomas (both lobular and ductal) (24,52) and early stage breast cancer (stages 0 and I) (49,53).

To be an effective marker, gene panels in methylation studies must include genes that are methylated specifically in cancer and not in normal tissue. Fackler et al (24) looked at the promoter methylation of a panel of genes (*RASSF1A, CCND2, TWIST, HIN1*) in samples obtained from invasive carcinoma and normal tissue adjacent to the tumor. They found that promoter methylation was more frequently detected in tumor than in normal tissue, though low levels of methylation were detected in normal control samples. In another study (54), normal tissue samples from the quadrant opposite of the primary tumor (n=12) showed methylation of each of the 23 genes examined, except for *CDKN2*. ROC curve analysis showed that a panel of 4 of these 23 genes (*CCND2, RASSF1A, APC* and *HIN1*) was able to distinguish between invasive carcinomas (n=66), fibroadenomas (n=31) and normal tissue (n=12) (54). Despite the

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fact that low levels of methylation were found in normal tissue, ROC curve analysis was still able to distinguish between normal samples and those with cancer.

A possible explanation for the methylation seen in some controls included in these studies is that, although not cancerous, the tissue samples may actually not have been ‘normal’. Alternatively, there may be a threshold for methylation to affect gene expression and lead to a growth advantage. A better understanding of methylation frequencies detectable in ‘normal’ tissue is needed and will aid in the selection of the appropriate source for normal tissue (i.e. proximal to the tumor, from the contralateral breast etc.) to use as control in comparative studies.

Distinguishing Between Benign and Malignant Disease

Benign breast diseases (BBD) are a diverse group of lesions which are poorly understood. BBD is an established risk factor for breast cancer, although the magnitude of the subsequent breast cancer risk remains controversial (55), partly due to the heterogeneity among types of BBD. Irrespective of its preneoplastic potential, it is important to consider BBD when assessing a potential breast cancer screening tool. BBD is extremely common: for example, post-mortem studies have estimated that one in two women develops fibrocystic disease and one in five, fibroadenoma, during her lifetime (56). Mammography is not always able to distinguish between cancer and BBD; this often requires a biopsy. A serum detection marker able to distinguish between benign and malignant breast tumors could reduce the number of breast biopsies and therefore have important public health value.

Studies testing the ability of promoter methylation profiles to distinguish between benign and malignant disease have led to mixed results (47,48,54,57-59). A study including women with invasive (n=24), *in situ* (n=10) and benign disease (n=8), as well as healthy controls (n=20) found that promoter methylation of three genes (*APC, RASSF1A, DAPK*) was detectable in DNA obtained from both *in situ* lesions and invasive samples at all tumor stages. No methylation however, was found in the controls or benign breast disease patients (53). In a different study, using a panel of genes including, *BRCA1, p16*^{INK4A}, *ESR1, GSTP1, TRβ1, RARβ2, HIC1, APC, CCND2* and *CDH1*, it was found that fibroadenomas (n=10) had patterns of methylation that were similar to that seen in breast cancer cases (n=54), with the exception of *CDH1*, which was more frequently methylated in cases than in benign breast disease. *CDH1* is known to be involved in cell adhesion and tumor progression (60), so it may have high specificity for invasive disease. Eighty-five percent of breast cancers and 70% of fibroadenomas had methylation of at least one of the genes in the panel with half of the cases having methylation in three or more genes (47).

In a study using breast tissue samples obtained by FNA biopsy from women with benign and malignant tumors (n=27) and unaffected women (n=55), a panel containing *RASSF1A, RARβ2, APC* and *CCND2* found that DNA from BBD lesions had an intermediate level of methylation, when compared to breast cancer cases and healthy controls (58). Promoter methylation (especially of *APC* and *RASSF1A*) was also found to be more frequent in healthy women predicted to have a high risk of breast cancer (using the GAIL model), than those predicted to have a low/intermediate risk. Further, using three of these same genes (*RARβ2, RASSF1A* and *CCND2*), in a study of 36 BBD, 21 *in situ* carcinoma and 45 invasive carcinoma, Pu et al (59) found there was an increase in the frequency of promoter hypermethylation from benign (42% had methylation of at least one of the three genes) to *in situ* carcinoma (76%) and invasive carcinoma (96%).

These have been small studies using variable gene panels on a wide range of benign conditions that are usually not specified. Further, the age of the subjects participating in these studies was not reported. This could have important implications on the interpretation of the results, since

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the methylation of tumor suppressor genes in benign breast epithelium has been shown to increase with age (61). Additional research is needed to assess the ability of promoter methylation analysis to distinguish malignant from benign conditions, with distinctions made between the type of BBD being studied and control for potential confounders such as age.

Detecting Lobular Carcinomas

Invasive lobular carcinoma (ILC) accounts for approximately 14% of all invasive breast cancers (62) and its incidence is rising (63). MRI appears to be a more efficient tool to detect ILC than mammography (63), which besides being inefficient in the detection of ILC is also unable to distinguish between ILC and invasive ductal carcinoma (IDC) (64). Promoter methylation may prove to be a useful tool to improve the detection of ILC.

Fackler et al (46) and Pu et al (59) conducted studies comparing the methylation patterns in ILC and IDC. They found that, overall, the two histological types had similar frequencies of methylation of each of the following genes; *RASSF1A, HIN-1, RARβ, CCND2, TWIST* (46); *RARβ2, RASSF1A, CCND2* (59). The study by Fackler et al (46) however, showed that the same panel of genes had variable sensitivity for the different tumor types: 69% for LCIS, while having a much higher sensitivity in ILC, DCIS and IDS (100%, 95% and 100% respectively).

A later study carried out by Bae et al (65) included 60 ductal, 30 lobular and 19 mucinous invasive breast carcinomas and 8 normal tissue samples obtained from reduction mammoplasty. Using a panel of 12 genes, the authors found that all invasive tumors had at least 3 genes with methylated promoters. They also found that, compared to IDC, mucinous and lobular cancers had a significantly higher mean frequency of methylation. However, the distribution of methylation frequency and number of genes methylated per case showed a significant degree of overlap among diagnostic subgroups. Nevertheless, the high frequency of promoter methylation seen in ILC supports a potential role for promoter methylation analysis in improving the sensitivity of lobular carcinoma detection.

Detection of Promoter Methylation in Circulating DNA

Promoter hypermethylation has also been detected in the serum/plasma of breast cancer cases (Table 2) (66-69). The results in tumor and blood samples from the same patients show good concordance (Table 3, mean overall concordance is 84%) (53,70-73). In a study conducted by Hoque et al (66), an analysis by disease stage showed that an accumulation of methylation occurs as the disease progresses. This study included a panel of four genes (*APC, GSTP1, RASSF1A* and *RARβ2*). Thirty-three percent of stage I/II (8 of 24 patients) and 65% of stage III/IV (43 of 66 patients) plasma samples showed methylation of at least one gene ($p=0.007$). In a study of 34 women with breast cancer, including 8 with BBD and 20 controls, Dulaimi et al (53) found that methylation was detectable in the serum of patients with early invasive and pre-invasive disease, while not detectable in normal serum samples, showing specificity of the markers.

Table 3 shows that in general, the sensitivity of a given gene panel in circulating DNA is slightly lower than the same panel in DNA obtained directly from the tumor. Like studies of tumor DNA, studies of serum DNA have included a variety of gene panels, with some overlap between studies. These panels have shown variable sensitivity with one 4 gene panel (*GSTP1, RARβ2, RASSF1A* and *APC*) having a sensitivity of 62% (66), while a different 4 gene panel (*RUNX3, p16, RASSF1A, CDH1*) had a greater sensitivity of 79% (69). This indicates the importance of gene selection in the sensitivity of the assay.

Because blood collection is a minimally invasive procedure, these studies tend to include more controls than their tissue based counterparts, but the numbers of controls used in each study is

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still low. The study including the greatest number of control samples (n=38) (66) found low levels of methylation for 2 out of the 4 genes investigated (*RASSF1A*, 5% and *RARβ2*, 8%). The significance of this methylation is unclear and requires further investigation.

The studies reviewed here have used highly variable amounts of DNA though often the precise amount used in the analysis was not reported. Many studies report only the volume of DNA used, rather than the concentration (66-68). Still others report a range or maximum amount of DNA used in each assay, in these studies 0.05 - 2μg (53,70-72). Reporting of the amount of DNA used in each assay and the volume of serum/plasma the DNA was obtained from will aid in the design of further studies using serum/plasma samples and clarify the minimum amount of DNA required for successful detection of aberrant changes in methylation patterns.

Limitations of Previous Studies and Need for Further Research

Pepe (74) and others (75) have suggested steps for the evaluation of new diagnostic markers. Each phase of the evaluation has its own study design and statistical measures (e.g., true positive rate (TPR) and false positive rate (FPR)) to assess the usefulness of the assay. Evaluation begins with the identification of potential markers using convenience samples. The next step is to conduct population-based case-control studies testing whether the marker are able to detect established disease. An important aspect of these studies is to use controls arising from the same population as the cases. Studies conducted to date however have included either no controls or only “convenience” controls that may not be comparable to the cases with respect to other characteristics. Further, the recommended sample size for adequate precision in calculating a true positive rate of 0.80 with a standard error of 0.05 and a false positive of 0.01 as no greater than 0.03 is 110 subjects without cancer and 70 subjects with cancer (75). Table 1 shows that many studies did not meet these criteria. These studies also did not account for potential confounders, such as age, in the analysis.

Another consequence of the small number of controls included in studies conducted to date is that our knowledge of normal patterns of promoter methylation is limited. A study including fine-needle aspiration biopsies from 55 unaffected women detected promoter methylation of *RARβ2* (9%), *APC* (26%), *H-cadherin* (17%) and *RASSF1A* (37%) (58). Lewis et al (58) also showed that methylation frequency increased with risk, as calculated by the Gail model. A recent study of 109 asymptomatic high-risk women, found frequent methylation of *RARβ* (70%), *p16* (29%), *HIN-1* (21%) and *PRA* (77%) and that this was associated with abnormal Masood cytology (76). Thus, promoter methylation may not be specific to cancer *per se*, but rather part of an accumulation of changes in DNA that occur over the course of a lifetime, eventually contributing to tumor development. Thus, when using ‘normal’ tissue samples, it is important to consider the source of the ‘normal’ tissue e.g. ‘normal’ tissue proximal to the tumor, ‘normal’ tissue from reduction mammoplasty, etc. This will help insure that meaningful case-control comparisons are being conducted.

Promoter hypermethylation has been identified as a potential marker and been shown to be able to detect established breast cancer. Following the path described by Pepe (74), the next step in the evaluation of promoter methylation is to conduct case-control studies nested within prospective cohorts to determine how well it is able to detect pre-clinical disease. Such prospective studies are expensive, require large sample sizes and long follow-up for a sufficient number of cases to be observed. Biological samples collected prospectively from cases are also very valuable and only small sample volumes are usually made available to study any given hypothesis. In light of these considerations we review below the questions that remain to be addressed with regard to the evaluation of the potential of methylation analysis for breast cancer screening.

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Need for Standardization of Methods for Methylation Analysis

Though a number of studies have been conducted in subjects with established breast cancer, methylation frequencies of genes measured in different labs and in different sample types have been variable and often not reproducible. This is largely due to 4 factors: 1) Variable methods of methylation analysis are used in different studies, 2) Gene panels are not consistent across studies, 3) If the same genes are used, often different promoter CpG sites are used and 4) sources of DNA are variable from study to study (i.e. serum, plasma, tissue, biopsy etc.).

The first issue to be addressed is the selection of the optimal method for methylation analysis. Optimization for small sample volumes (and therefore a small amount of DNA template) should be the focus, to allow for the use of samples obtained from existing prospective studies which are needed to assess the ability of promoter methylation patterns to detect pre-clinical disease. This will require the determination of the absolute sensitivity of the different methods. The absolute sensitivity of an assay is the minimum quantity of target DNA required for successful amplification and detection (77). To accomplish this, it is suggested here that criteria for publication of methylation data be standardized and include the requirement for confirmation of methylation results from non-sequence based methods (i.e. MSP, and QMSP) by bisulfite sequencing (the gold standard) for a subset of samples. It is also suggested that the amount of DNA used in each assay and the coefficients of variation (CV) for any repeat measures, be reported.

Reproducibility of methylation results is an area of great importance, one that has not been sufficiently addressed in the current literature. Methylation frequencies have largely not been reproducible across studies. This variability may be reduced with the standardization of methods and reporting of results. One study designed to specifically examine the reproducibility of the PMR (percent of fully methylated DNA found in a sample), 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%) (78). In a recent study, methylation results using a nested QMSP method (QAMA) on DNA obtained from micro-dissected cells from formalin-fixed and paraffin-embedded tumor tissues (n=13) was found to have a good correlation with sequencing results (R=0.982). To our knowledge no studies have reported the reproducibility of measurements obtained from serum or plasma samples.

Because no single gene has been found to be methylated in all breast cancers, it is necessary to use a panel of genes. The variability in the genes included in each panel makes it difficult to compare or combine the results of different studies and to infer how promoter methylation would fare as a screening tool. Further, though two studies may have included the same genes in their panels, they have not necessarily probed for the same CpG sites within the promoter. This adds another layer of variability between studies and there is no concensus or criteria for the selection of CpG sites within a promoter.

Regarding panel selection, up to now it has been based largely on the candidate gene approach, using genes that have a known involvement in carcinogenesis. Methylation of these genes can be found in many other forms of cancer and is not specific to breast cancer. There are genes however, that may have an increased role in breast cancer specifically, such as *GSTP1*; which is known to be involved in hormone related cancers (79), *BRCA1*; a known player in the family history of breast cancer such that patients with methylated *BRCA1* having a similar phenotype to those with *BRCA1* mutations (80) and *ERS1* and its associated genes because of the known role of estrogen in breast carcinogenesis (81). The inclusion of these genes may help improve the specificity of a gene panel for breast cancer.

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It will also be important to understand those factors that influence methylation analysis, such as the source of DNA (i.e. serum versus plasma), sample volume, sample handling, storage temperature and duration and freeze/thaw cycles. For example, variation in the amount of DNA obtained from serum and plasma has been shown. The major difference between serum and plasma is the presence of clotting factors (and associated proteins) in plasma. It appears that serum tends to contain approximately 6-fold more DNA than plasma does. Much of this DNA, however, could come from the normal DNA of contaminating leukocytes (82). Further, large prospective studies needed to test the diagnostic potential of these markers requires long periods of sample storage as cases are accrued through follow-up. The effect of this long-term storage on DNA methylation also needs examining. The Early Detection Research Network (EDRN) of the National Cancer Institute (NCI) has made understanding those factors that may influence methylation analysis, part of their focus (83).

Summary and Conclusion

Localized breast cancer has a five-year survival rate of 98%. However, when diagnosed after the tumor has metastasized, the survival rate decreases drastically to 27% (1). These results point to the benefit of screening and early detection. Given that mammography sensitivity is as low as 50% in some groups of women, the potential for methylation markers in circulating DNA, to complement the results of mammography in breast cancer detection and diagnosis, deserves further exploration (84) and has been the focus of this review.

Changes in promoter methylation status are frequent events that occur early in the tumorigenic process and are detectable through minimally invasive measures. A number of cancer-related genes have been found to be frequently methylated in breast cancer. These markers show promise for distinguishing between malignant disease and benign disease or normal tissue, and they may be able to improve the detection of lobular carcinomas. Furthermore, the combination of this minimally invasive procedure with mammography could improve the sensitivity of tumor detection in women with high breast density, a characteristic that is associated with an increased breast cancer risk (85) and reduced sensitivity of mammography (10-12).

Additional questions of interest include whether methylation patterns vary with ER/PR status (81,86,87), and in *BRCA* mutation carriers or in familial breast cancers (76,88). Ideally, to be a successful screening tool, a marker would be able to detect breast cancer, regardless of its receptor status, origin or subtype. The selection of the genes to form the diagnostic panel will likely determine how successful promoter methylation is in identifying breast cancer and the type of breast cancer it is detecting.

Currently, most studies select genes based on known gene function and methylation frequency. As we gain a better understanding of the methylome, a map of genome-wide, tissue-specific patterns of methylation (89) this is expected to change. Micro-arrays designed specifically for bisulfite treated DNA are available but currently are not optimized for high-throughput analysis and account for only 0.1% of the total CpG sites in the human genome (89). The future of methylation analysis will likely involve a combination of isolation of the methylated fraction of DNA either using MBD proteins (Methylated-CpG Island Recovery Assay (MIRA)) or immunocapture (Methylated DNA Immunoprecipitation on Chips (MeDIP-chip)) methods and next generation microarray or sequencing technologies (89). The optimal method for analysis however, will ultimately depend on the research goals of the analysis since currently no one method is able to balance the need for quantitative accuracy, sensitive detection, local versus global information and automation (90).

Studies in well defined populations, including appropriate controls and larger numbers are needed to further evaluate the potential of DNA methylation to improve current breast cancer

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screening strategies. In order to successfully conduct these studies, optimization and standardization of methylation detection assays that can be used on small volumes of serum/plasma frozen for extended periods of time are needed.

Acknowledgments

Support: Susan G. Komen for the Cure BCTR0707521, DOD Pre-doctoral Training Grant BC060891, National Cancer Institute R01 CA098661, the National Cancer Institute Cancer Center Grant CA-016087, and the National Institute of Environmental Health Sciences center grant ES-000260.

Paul Cairns is funded by the NIH Early Detection Research Network 1 U01 CA111242

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Table 1

Characteristics of Selected Studies of Promoter Methylation Detected in Tissue

Reference	Type of tumor (Number of cases)	Genes	Methylation Frequency	Coverage[A]
Fackler et al (46)[B]	LCIS (n=13)	RASSF1A HIN-1 RARβ CCND2 TWIST	62% 46% 46% 23% 23%	69%
	ILC (n=19)	RASSF1A HIN-1 RARβ CCND2 TWIST	84% 79% 21% 32% 16%	100%
	DCIS (n=44)	RASSF1A HIN-1 RARβ CCND2 TWIST	75% 68% 48% 32% 27%	95%
	IDC (n=27)	RASSF1A HIN-1 RARβ CCND2 TWIST	70% 60% 41% 52% 56%	100%
Fackler et al (24)[C]	Cases (n=19) (n=21) (n=21) (n=21)	RASSFIA TWIST CCND2 HIN-1	68% 67% 57% 57%	84%
	Controls (n=28) (n=18) (n=14) (n=16)	RASSFIA TWIST CCND2 HIN-1	7% 6% 14% 7%	
Parella et al (47)[B]	Cases (n=54) (44 IDC, 10 ILC)	BRCA1 P16 ESR1 GSTP1 TRβ2 RARβ2 HIC1 APC CCND2 CDH1	17% 18% 46% 13% 28% 20% 48% 28% 11% 39%	85%
	BBD (n=10)	BRCA1 P16 ESR1 GSTP1 TRβ2 RARβ2 HIC1 APC CCND2 CDH1	20% 20% 40% 0 0 0 30% 10% 0 0	
Jeronimo et al (48)[B]	Cases (n=27)	CDH1 GSTP1 BRCA1 RARβ2	66% 58% 40% 34%	88%
Tao et al (49)[C]	Cases (n=803)	CDH1 p16 RARβ2	20% 26% 28%	60%

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Reference	Type of tumor (Number of cases)	Genes	Methylation Frequency	Coverage[A]
Shinozaki et al (50)[B]	Cases (n=151)	RASSF1A APC TWIST CDH1 GSTP1 RARβ2	81% 49% 48% 53% 21% 24%	Not Calculated (at least 81%)
	Controls (n=10)	All genes	0	
Li et al (51)[B]	Cases (n=193)	RARβ2 CDH1 ESR1 BRCA1 CCND2 p16 TWIST	26% 80% 84% 41% 11% 14% 59%	Not Calculated (at least 84%)

[A]Coverage, percentage of cases having methylation of at least one gene in the given gene panel (i.e. coverage of 100% means that all samples had methylation of at least one gene in the study's panel); LCIS, lobular carcinoma *in situ*; ILC, invasive lobular carcinoma; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; BBD, benign breast disease.

[B]Methylation analysis was conducted using methylation specific PCR (MSP).

[C]Methylation analysis was conducted using quantitative real-time methylation specific PCR (QMSP).

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Table 2

Characteristics of Selected Studies of Promoter Methylation Detected in Circulation

Reference	Sample Type and Size	Genes	Methylation Frequency	Coverage [A]
Hoque et al (66)[B]	Plasma Cases (n=47)	GSTP1 RARβ2 RASSF1A APC	26% 26% 32% 17%	62%
	Plasma Healthy Controls (n=38)	GSTP1 RARβ2 RASSF1A APC	0 8% 5% 0	
Müller et al (67)[B]	Serum Cases Primary tumors (n=26)	ESR1 APC HSD17β4 HIC1 RASSF1A	27% 23% 12% 39% 23%	Not Calculated
	Recurrent breast cancers (n=10)	ESR1 APC HSD17β4 HIC1 RASSF1A	70% 80% 30% 90% 80%	
	Healthy Controls (n=10)	ESR1 APC HSD17β4 HIC1 RASSF1A	0 0 0 10% 10%	
Papadopoulou et al (68)[B]	Plasma Cases (n=50)	RASSF1A ATM	26% 14%	36%
	Healthy Controls (n=14) (n=9)	RASSF1A ATM	0 0	
Tan et al (69)[C]	Serum Cases Metastatic (n=19)	RUNX3 p16 RASSF1A CDH1	47% 37% 42% 0	79%
	Control (n=10)	All genes	0	

[A]Coverage, percentage of cases having methylation of at least one gene in the given gene panel (i.e. coverage of 100% means that all samples had methylation of at least one gene in the study’s panel).

[B]Methylation analysis was conducted using quantitative real-time methylation specific PCR (QMSP).

[C]Methylation analysis was conducted using methylation specific PCR (MSP).

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Table 3

Promoter Methylation Concordance Between Paired Tissue and Circulating Samples

Reference	Sample Size	Genes	Methylation Frequency Tissue/Blood[B]	Coverage[A] Tissue/Blood	Concordance
Dulaimi et al (53)	Cases (n=34)	APC RASSF1A DAPK	47%/29% 65%/56% 50%/35%	94%/76%	81%
	Controls (n=20) BBD (n=8)	All genes	0		
Sharma et al (70)	Cases (IDC) (n=36)	p16 p14 CCND2 Slit2	44%/36% 47%/36% 27%/25% 58%/58%	86%/83%	89%
	Controls (n=4)	All genes	0		
Mirza et al (71)	Cases (IDC) (n=50)	TMS1 BRCA1 ESR1 PRB	24%/24% 26%/22% 66%/48% 64%/46%	72%/64%	88%
	Controls (n=5)	All genes	0		
Shukla et al (72)	Cases (IDC) (n=20)	RASSF1A RARβ	85%/75% 10%/0	85%/75%	79%
Hu et al (73)	Cases (IDC) (n=36)	p16 CDH1	11%/8% 25%/20%	31%/25%	82%

[A]Coverage, percentage of cases having methylation of at least one gene in the given gene panel (i.e. coverage of 100% means that all samples had methylation of at least one gene in the study's panel); Concordance, between paired samples.

[B]In all cases methylation analysis was conducted using methylation specific PCR (MSP).