

Strategic Approach to Validating Methylated Genes as Biomarkers for Breast Cancer

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Abstract

Although the potential of biomarkers to aid in the early detection, diagnosis, prevention, and treatment of breast cancer is broadly recognized and numerous biomarker candidates have been reported in the literature, few molecular markers have been adopted into clinical use to date. To address this lack of translation of biomarkers from the bench to clinical practice, the Cancer Biomarkers Research Group in the Division of Cancer Prevention of the National Cancer Institute organized a meeting, "Strategic Discussion on Biomarkers for Breast Cancer," which was held at the Fred Hutchinson Cancer Research Center on September 14, 2008. Participants included industry leaders, basic and physician scientists, and National Cancer Institute program staff. The objectives of this strategic discussion were to define clinical uses and needed performance characteristics of biomarkers; to identify novel approaches to discover and validate breast cancer biomarkers, particularly those with improved chances of being clinically useful; and to identify candidate DNA methylation markers that could be taken forward for validation. Participants presented and recommended methylation biomarkers suitable for initiating collaborative projects to evaluate the markers for future clinical application. This commentary summarizes their discussions and recommendations and the rationale for initiating specific projects to validate DNA methylation biomarkers of breast cancer. *Cancer Prev Res*; 3(1); 16–24. ©2010 AACR.

Introduction

Although breast cancer death rates have been dropping steadily since 1990, it remains the second leading cause of cancer deaths in women. According to the American Cancer Society, there were ~40,460 breast cancer deaths in the United States in 2007. Reducing suffering and death from breast cancer depends on improving screening, diagnosis, prognosis, prediction, and treatment. Biomarkers have the potential to contribute to all these areas, either alone or in conjunction with imaging approaches. But few breast cancer biomarkers thus far have moved out of the discovery phase into validation and clinical usage. Reasons for this lack of translation include the heterogeneity of breast cancer, general misconceptions about the biomarker validation process, and limited understanding about the requirements for a biomarker to be clinically useful and to be commercialized.

Numerous breast cancer biomarkers have been and continue to be reported, but the challenge is to determine which markers are useful for clinical diagnosis and how to transfer these markers and relevant technologies to clinical

application. This requires biomarkers to be critically evaluated for how they were discovered, including specimens used, the experimental design, technologies and data analysis methods used, and the specific clinical application (screening, diagnosis, prognosis, or prediction). As an initial step in this process, the NCI-sponsored "Strategic Discussion on Biomarkers for Breast Cancer" meeting focused on one type of biomarker, DNA hypermethylation, which has received numerous experimental evidence as a promising biomarker likely to move from the discovery phase to clinical use. Participants in the meeting were asked to identify methylation biomarkers, specify clinical samples used, present enabling technologies, and to emphasize clinical usefulness of these methylation markers. This report summarizes the meeting presentations, discussions, and recommendations as well as the emerging rationale for initiating a project to validate DNA methylation biomarkers for breast cancer early diagnosis.

Breast Cancer Biomarkers: Clinical Issues

Any discussion of biomarkers for breast cancer, or for any other cancer, must include the clinical context in which they are to be used. The performance characteristics a marker needs to have and the type of specimen in which it will be measured depends on whether it will be used for screening, risk assessment, diagnosis, prognosis, or prediction. Available imaging methods must also be considered, as their performance in part dictates the performance of a clinically useful biomarker.

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Screening and early detection

A screening marker is used in asymptomatic people to detect a disease or condition at an early stage (1, 2). Breast cancer screening is used for the general population with a goal of finding the disease in people who do not show any symptoms. The intent is to detect the cancer early when it is still very small and noninvasive, and there is a much better chance of successful treatment. A screening test must be relatively inexpensive and minimally invasive. A screening biomarker must be measurable in a readily accessible body fluid. Future useful screening biomarkers should be considered in the context of currently widely used methods, such as mammography and self-examination.

Currently, there is no molecular biomarker panel that is used in clinical practice for breast cancer screening or early detection. Mammography is standard practice in the United States and is considered the current "gold standard" for breast cancer screening and early detection. Screening mammography uses low-dosage X-ray imaging to detect tumors and other abnormalities in women who have no symptoms or observable breast abnormalities. Mammography is also used for diagnosis to investigate suspicious breast changes, such as breast pain, a breast lump, an unusual skin appearance, nipple thickening, or nipple discharge. Although mammography is currently the main modality for breast cancer screening and early detection in the United States, it has several potential harms, which can cause anxiety and unnecessary invasive diagnostic procedures (3). These include discomfort, potential damages from X-ray, and a relatively high rate of false positives (20-56% after 10 mammograms among women 40-49 years of age). In addition, the sensitivity of mammography is about 78% to 87%; thus, >10% of cancers will be missed by mammography (4). The accuracy of mammography also depends on breast density. It is more difficult to accurately detect breast cancer in women with dense breast tissue that could cover tumors from a mammogram, such as among younger women who more frequently have aggressive disease (5, 6). Therefore, new screening approaches are needed for the early detection of breast cancer in all age groups. In addition, the cost of mammography is a concern in certain developing countries; if molecular biomarkers, which have better or equivalent sensitivity to early detect breast cancer, are developed, these markers could be used as initial or routine diagnosis before mammography.

For a biomarker to be widely accepted to screen asymptomatic women for breast cancer in the United States, it must have sensitivity and specificity comparable or superior to that of mammography. No biomarkers or panel of biomarkers have this level of performance yet. Biomarkers that could be used in conjunction with mammography to decrease the number of false negatives (improve sensitivity) would be beneficial if it did not result in an increase in false positive (decreased specificity). A biomarker with high specificity and reasonable sensitivity might have a role in screening in developing countries where there is currently no screening program and that cannot afford mammography.

Risk assessment

Risk assessment uses biomarkers and other factors to estimate a woman's risk to develop breast cancer. Risk assessment and stratification are related and, sometimes, interchangeable. The latter is more related to classifying patients into low, moderate, or high risk of developing an invasive breast cancer, determined by known clinical risk factors, and is more related to predicting benefit of a treatment relative to possible risks of side effects. Biomarkers can be used to select high-risk people or targeted populations for preventive interventions, specific screening, and early diagnostic strategy and prevention.

Inherited breast cancer accounts for about 5% to 10% of all breast cancers. Women with inherited mutations in *BRCA1* and *BRCA2* are at high risk of developing both breast and ovarian cancer. In addition, several genetic modifiers for *BRCA1* and *BRCA2* have been evaluated over 8,000 women, and one, *RAD51*, has been confirmed (7). Current risk assessment models for sporadic breast cancer are not based on molecular biomarkers, but are built on other risk factors. For example, the Breast Cancer Risk Assessment Tool¹ uses medical history, age, age of the first menstrual period, age of the first child birth, breast cancer of the first-degree relatives, and biopsy to calculate approximately a woman's risk to develop invasive breast cancer. This tool is not for assessing breast cancer risk for women who have already had a diagnosis of breast cancer.

Diagnosis

A diagnostic marker is used in people with signs or symptoms to aid in assessing whether they have a condition (1, 2). Women with an apparent abnormality on mammogram or a palpable lump might undergo biopsy to determine whether they have breast cancer. Biomarkers could be used in women who have a positive finding to increase the accuracy of distinguishing breast cancer from a benign lesion. This could reduce the number of unnecessary biopsies. Biomarkers for this purpose would need to have very high sensitivity (that is, the percentage of people who test positive for a specific disease among a group of people who have the disease should be closer to 100%) so that no cancers are missed; high specificity (that is, the percentage of people who test negative for a specific disease among a group of people who do not have the disease) is less of a problem as these women would have gone for biopsy anyway. In addition, biomarkers are needed to diagnose breast cancer in patients with abnormal symptoms. For example, spontaneous nipple discharge is a risk factor for breast cancer, but only 1% to 23% of spontaneous nipple discharge result from a neoplasia. Mammary ductoscopy image visualization is not reliable to distinguish benign from malignant, and cytologic evaluation of ductal cells is also not reliable. Therefore, molecular markers that distinguish benign conditions from cancer could also save many women from unnecessary surgery (8).

¹ <http://www.cancer.gov/bcrisktool>

Prognosis

A prognostic marker is used in subjects with a condition to predict subsequent outcomes, such as disease recurrence or prognosis. For clinical diagnosis and prediction of the development of benign lesions to malignance, currently used biomarkers primarily are histologic atypia, cytologic atypia, cell proliferation, estrogen receptor (ER) expression, estradiol and related hormones, mammographic density, and several molecular biomarkers (9). At this time, there are no "omics"-based molecular biomarker panels for predicting benign lesions to cancer. Atypia whether diagnosed by cytology using fluid from nipple aspiration fluid (NAF) or histology using tissue specimens from fine needle aspiration (FNA) predict similar relative risk of 4% to 5% for breast cancer (10–12). Expression of ER- α and proliferation antigen Ki-67 have been studied by using morphometric image analysis and dual-labeled immunofluorescence in nonatypical hyperplasia or hyperplasia of usual type foci and in surrounding normal lobules. Patients who progressed to breast cancer showed significantly higher ER- α and Ki-67 expression in their hyperplasia of usual type foci compared with those who did not progress to cancer (13). Thus far, these markers have been studied in a limited number of patients and their accuracy of predicting development of malignance should be evaluated and improved.

Oncotype DX, a gene expression biomarker panel, has been used as a clinical test since 2004 for both prognosis and prediction of recurrence. Oncotype DX is a diagnostic test that estimates both the likelihood of breast cancer recurrence in women with newly diagnosed, early-stage invasive breast cancer, and the potential benefit from chemotherapy. The assay is done by using formalin-fixed, paraffin-embedded tumor tissue to analyze the expression of a panel of 21 genes. The results are provided as a recurrence score. The development of OncotypeDX into a commercial test provides several of valuable lessons to others seeking to commercialize their biomarker. An important first step was to take into account the needs of patients, physicians, and payers. After identifying that there was a clear need for biomarkers to select node-negative and ER-positive (ER+) breast cancer patients who could get benefits from chemotherapy treatment, an overall strategy was established to perform multiple studies. The approach included gene expression research, technical development, analytic and clinical validation, and accurate and standard study design, performance, and analysis. Clinical validations are a key step for biomarkers to be transferred to clinical usage. With the successful establishment of the 21-gene expression-based recurrence score algorithm, high throughput trial implementations and high throughput

Table 1. Strengths and limitations of methods for sampling breast specimens

Methods	What is sampled	To whom	Analysis	Purpose	Strength	Limitation	Text reference
NAF	Breast epithelial cells	Asymptomatic women	Cytology: proliferation and atypia	Risk assessment/stratification	Minimally invasive	Insufficient cells for morphologic assessment	Fabian et al., 2005 (22)
DL	Breast epithelial tissues/cells	Asymptomatic women	Cytomorphology: atypia	Risk assessment/stratification	Minimally invasive	1/3 of ducts accessible by DL; low sensitivity for detection	King et al., 2006 (14); Fabian et al., 2005 (22)
RPFNA	Breast epithelial tissues/cells	Highest density of precancerous lesions	Hyperplasia, atypia	Short-term risk	Minimally invasive	Unknown location of atypia	Fabian et al., 2005 (22)
FNAB	Breast epithelial tissues/cells	Asymptomatic women	Hyperplasia, atypia	Diagnosis	Minimally invasive	Time consuming, intolerant	Chaiwun et al., 2007 (15); Shidham et al., 2007 (20); Fabian et al., 2005 (22)
CNB	Epithelial tissues/cells	Subjects in prevention trials	Hyperplasia, atypia	Risk assessment and prevention trials	Large number of epithelial cells	Difficult to accrue subjects	Fabian et al., 2000 (11)

Abbreviations: RPFNA, random periareolar FNA; FNAB, fine-needle aspiration biopsies.

assays were developed. For a successful trial, all these requirements must be achieved in a proper and efficient manner, using specimens, Internal Review Board approvals, pathology reviews, reverse transcription-PCR analysis, quality control, and data analysis. Clinically used biomarkers must also follow standard good practices as set forth in the Clinical Laboratory Improvement Amendments.

Participants in this NCI strategic discussion thought that a critical area where biomarkers could play an important role is in the prediction of which benign or precancerous lesions will progress; specifically which atypical hyperplasia, atypical ductal hyperplasia, or atypical lobular hyperplasia will progress to ductal carcinoma *in situ* (DCIS), to lobular carcinoma *in situ*, or to invasive breast cancer; and which DCIS or lobular carcinoma *in situ* will progress to invasive cancer.

Specimen for Breast Cancer Biomarkers

Choice of specimen is an important part of both biomarker discovery and its potential usefulness in a clinical assay. Knowing the types of specimens used for discovering a biomarker could help in developing and choosing noninvasive sampling methods and retrieval of breast epithelial cells for diagnosis. Sources for breast cancer biomarkers include body fluids, such as sera, urine, saliva, ductal lavage (DL), nipple aspirate, and fine needle and core needle biopsy (CNB; Table 1; refs. 11–22). FNA typically involves 8 to 10 aspirations per breast using a 20- to 21-gauge needle. This process can provide ~10,000 epithelial cells, but the samples also frequently contain macrophages and fibroblasts as well as contamination of red cells (15–18). Cell yield could be increased by rinsing the needle in a fixative and methods that produce higher specimen yield have been studied (19, 20). A CNB typically removes small samples of breast tissue by using a 16-, 14-, or 11-gauge hollow core needle (21). The palpation-guided biopsy is for palpable lesions; while one hand fixes the lesion, the other performs the biopsy. Stereotactic mammography uses computers to pinpoint the exact location of a breast mass based on mammograms (X-rays) taken from two different angles. Ultrasound-guided CNB uses ultrasound imaging to help guide the site of the abnormal growth. NAF is to elicit nipple fluid by breast massage and suction-aspiration at the nipple; NAF samples include hormones, proteins, and growth factors, generally paucicellular. DL is obtained by using a breast aspirator to apply mild suction to the nipple, or by inserting a cannula into ducts, to draw out tiny amounts of nipple fluid from the milk ducts. The overall average of DL cellularity is ~7,000, and the reproducibility of cytologic findings of the first and second lavage from same duct is ~47%. Although concerns have been brought regarding the difficulty of the procedure and discomfort of subject, studies have shown that DL could obtain cells sufficient for repeat biomarker analysis in 133 of 157 (84.7%) of ducts (9). The advantages of DL in biomarker monitoring are its cellularity and reproducibility. The potential promise of DL is to add precision to risk estimation in asymptomatic high-risk women, to

allow serial observation of breast epithelial response to preventive agents, and to possibly detect mammographically occult breast cancer. Breast tissue samples have been successfully used for preventive clinical trials (22).

Epigenetic Markers in Sporadic Breast Cancer

Sporadic breast cancer is a complex and heterogeneous disease in respect to biology, morphology, and clinical behavior as well as molecular alterations. It is not known how normal breast tissue or benign breast diseases progress to breast cancer and to what types of breast cancer. Accurately detecting different types of breast cancer and distinguishing benign diseases that are likely to progress to cancer from those that are unlikely to progress will improve early detection and preventive strategies. Data from omics-based research are a rich source for identifying potential new molecular tools and biomarkers. Aberrant DNA methylation is among the most common molecular alterations in human neoplasia and seems to be involved in the early stage of breast cancer carcinogenesis. Hypermethylation or hypomethylation can silence tumor suppressor genes and activate oncogenes, respectively, and thereby alter functional genes to promote abnormal cell growth. During this NCI-sponsored meeting, participants, including those in the Early Detection Research Network (EDRN), focused on this category of epigenetic markers to examine the potential usefulness of DNA methylation status in clinical specimens and in circulating body fluid for detecting sporadic breast cancer and predicating the likelihood of progression from benign disease or precancerous lesions to cancer. Studies have shown aberrant DNA methylation of various genes in invasive and noninvasive human breast cancers and in tumor-derived DNA in the blood, suggesting that these epigenetic markers may be useful for risk assessment and progression prediction.

A direct approach to discover DNA methylation markers for breast cancer is to examine the methylation status of functionally important genes in specimens from breasts of patients with cancer and those without. Underlying this method is evidence that alteration in the expression of functionally important genes, tumor suppressor genes, and oncogenes can initiate cancer development; most DNA methylation markers are not breast cancer specific; and noninvasive breast specimens (FNAs, nipple DL, and spontaneous nipple discharge) are available for molecular biomarkers research. This approach has identified several methylation markers that have potential to improve cancer diagnosis, risk assessment, and treatment (Table 2; refs. 23–33). Several genes, including *RASSF1a*, *Hln1*, *Cyclin D2*, have shown consistent association with breast cancer in several independent studies, although the magnitudes of these genes' methylation are variable. Furthermore, gene panels, instead of a single gene's methylation for breast cancer risk, have been studied. For example, the cumulative methylation index of a panel of genes (*RASSF1a*, *TWIST*, *HIN1*, *Cyclin D2*, *RAR3*, *BRCA1*, *BRCA2*, and *p16*) could detect cancer with a sensitivity of 62%, whereas

Table 2. Performance analysis of frequently methylated genes in breast cancer for clinical application

Gene	RASSFA1	Twist	Hin1	Cyclin D2	RAR β	APC1	BRCA1	BRCA2
Results								
Sensitivity	38.1	52.4	42.9	28.6	19.1	33.3	14.3	0
Specificity	97.4	89.5	98.7	100	98.7	96.1	100	96.1
MF of DCIS in Americans %	75	65	67	56	39	83	25	10
MF of DCIS in Koreans %	85	65	73	60	54	69	33	10
MP in BC %	68		58	65	39	49		
MP in Gail high risk %	35		20	18	15	25		
MP in Gail lower risk %	10		10	9	10	9		
MF in benign %	34			2.9	33			
MF <i>in situ</i> %	62			56	60			
MF in invasive %	64			71	64			
MF in LCIS %	62		46	23	46			
MF in ILC %	84		79	32	21			
MF in DCIS-1 %	88		79	29	36			
MF in DCIS-2 %	58		58	25	58			
MF in DCIS-3 %	67		50	39	50			
MF in IDC %	70		41	52	41			

(Continued on the following page)

the sensitivity of cytology for detecting cancer was 33% in the same set of DL samples from women undergoing mastectomy with or without breast cancer; however, the specificity of the panel was 83% compared with that of cytology, which is 99% (23). It seems possible that combining DNA methylation markers and cytology might increase sensitivity without decreasing specificity in diagnosis. Significant differences in DNA methylation signatures between certain benign breast conditions, such as papilloma and DCIS, compared with cancer have been shown (31, 32). Promoter hypermethylation of *RUNX3* was significantly higher in the neoplastic breast samples than in normal breast tissues (29). Hypermethylation of one or more of a five-gene panel (*RASSF1A*, *HIN-1*, *RAR- β* , *Cyclin D2*, and *Twist*) has been reported for early diagnosis (33). Risk assessment of low risk to high risk, as defined by the Gail model, is associated with a modest increase in the number and intensity of methylated genes. But progression to cancer was associated with an abrupt increase in the methylation of multiple functionally important genes (27, 28). Evidence of similar patterns of DNA methylation in discharge fluid, tissue, and ductal cells dislodged during ductoscopy provide the potential to use these specimens for DNA methylation-based molecular diagnosis. However, most of these studies have been conducted with different types of specimens or relevant small size samples and analyzed with various methods. Although these previous studies have provided initial evidence for using methylation markers for breast cancer, they cannot provide conclusions to decide how to further move these markers forward to primary status. A well-designed and organized prevalidation study is necessary to further evaluate these markers.

In addition to evaluating DNA methylation markers in specimens from breast tissues, accurately detecting tumor cell-specific aberrant DNA hypermethylation markers in sera or plasma, an easily accessible bodily fluid, is a highly promising approach to developing a noninvasive breast cancer diagnostic assay. The principle of discovering DNA methylation markers for breast cancer is based on evidence that blood from individuals with cancer is known to contain neoplastic DNA, which could come from circulating tumor cells or cell-free DNAs released from dead tumor cells. Currently, several studies have measured circulating tumor cells to predict progression and survival for metastatic breast cancer, few for early detection. This might be caused by the very limited number or nondetectable circulating tumor cells in presymptom patients. Altered DNAs by hypermethylation have been evidenced as an early cancer phenomenon; therefore, methylation biomarkers have high potentials to be used for early detection and prediction of precancerous disease to cancer. Several studies have shown concordance of DNA methylation patterns between blood and tumor. Genes that have shown promising results include *RASSF1A*, *HIN1*, *APC*, *GSTP1*, *RAR β 2*, *CTGF*, *NTRK2*, *CSR2*, *p16*, *TWIST*, *CYCLIND2*, *RUNX3*, *SFRP1*, and *REPRIMO* (34–42). When methylation of *APC*, *GSTP1*, *RASSF1A*, and *RAR β 2* were measured in plasma from 93 women with breast cancer and 76 controls, methylation of at least one gene resulted in a sensitivity of 62% and a specificity of 87% (36). In another study, methylation of *RASSF1A*, *APC*, and *DAP-K* was measured in 34 breast tumors and paired with preoperative serum DNA. One or more genes were methylated in 32 of 34 (94%) breast tumors; *RASSF1A* was hypermethylated in 22 of 34 (65%), *DAP-K* in 17 of 34 (50%), and *APC* in 15 of

Table 2. Performance analysis of frequently methylated genes in breast cancer for clinical application (Cont'd)

p16	CDH1	Era	Clinical application	Sample	Text reference	Comments
9.5			Diagnosis	DL	Fackler et al., 2006 (23)	Small size
100	8	10	Diagnosis	FNA	Lee et al., 2008 (24)	Need to study specificity and sensitivity
			Risk prediction	FNA	Euhus et al., 2008 (26)	Need to study specificity and sensitivity
			Categorization	FNA	Pu et al., 2003 (31)	Further study precancer progression to cancer
			Categorization	Tissue block	Fackler et al., 2003 (33)	Small sample size

Abbreviations: LCIS, lobular carcinoma *in situ*; MF, methylation frequency; MP, methylation prevalence; BC, breast cancer; ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma.

34 (47%) tumors. Twenty-six (76%) of the corresponding sera DNA from the cancer patients were positive for promoter hypermethylation, whereas no hypermethylation was observed in DNA of sera from normal healthy women and patients with inflammatory breast disease or nonneoplastic breast tissue specimens (34). A third example is a study of 33 early-stage breast cancer patients in which the methylation of tumor-related genes *RAR-ss2*, *MGMT*, *RASS-F1A*, and *APC* were determined. A concordance was found between gene hypermethylation detected in bone marrow, serum samples, and matched pair primary tumors (42). DNA methylation assays using body fluids, such as serum, are promising, but these results should be further evaluated in a systematic fashion with a larger number of specimens.

MicroRNAs are a new class of regulators of RNA translation, and aberrant methylation of genes encoding microRNAs has been reported. Aberrant hypermethylation was shown for *mir-9-1*, *mir-124a3*, *mir-148*, *mir-152*, and *mir-663* in 34% to 86% of cases in a series of 71 primary human breast cancer specimens (43).

The most common method used in these studies was methylation-specific PCR, including quantitative multiplex methylation-specific PCR, which allows for the detection of low levels of DNA methylation, being able to detect 1 methylated copy among 100,000 unmethylated copies (23, 44). But this technology can only detect CpG islands that are within the primer sequences and cannot detect CpG sites outside the methylation-specific primers. Several other methylation assays have been developed for different purposes, and each has certain advantages, limitations, and suitability. For example, a differential methylation hybridization assay uses methylation-insensitive restriction enzymes to cut unmethylated CpG islands, followed by PCR and hybridization to globally profile DNA methylation of ~50,000 CpG-rich islands in the human genome (45). Microarray methylation assessment of single samples uses a more efficient enzyme, McrBC, to cut unmethylated sequences. A newly developed digital MethyLight assay could more efficiently detect cancer-specific DNA hypermethylation in plasma samples from breast cancer

Table 3. Bias in studying molecular biomarkers/DNA methylation for breast cancer

Source of bias	Outcome	Factors for improvement	Text reference
Biology/heterogeneity of breast cancer disease	Imprecise diagnosis and prediction	Molecular diagnosis	Bertucci and Binbarum, 2008 (47)
Sampling	Inaccurate or fluctuating results	Reference samples	Collins et al., 2006 (48)
Assays	Incorrect measurement of methylation	Standardization and replication	Tost et al., 2007 (49)
Data analysis	Inaccurate estimation and conclusion	Proper experimental design and standard analysis	Sims et al., 2008 (50)

patients (46). Because different methods of detecting methylation have some different limitations and advantages, for a multisided, blinded prevalidation study, a consensus standard assay should be established for all performance sites.

Strategic Approach to Breast Cancer Detection

Because breast cancer is a complex and heterogeneous disease, accurately detecting breast cancers will likely require using multiple approaches and technologies. Biomarkers could become one of the tools for breast cancer detection if the markers have advantages over current detection technologies or add additional information that can allow for more accurate detection breast cancer, classification of subtypes, and prediction of cancer progression. Studies of DNA methylation for breast cancer could present certain bias, which is related to sampling, techniques, design, and data analysis (Table 3; refs. 47–50). To overcome various biases, a systematic approach is needed to identify a panel of true positive biomarkers from the large number of biomarkers reported every year. Methylation of dozens of genes in various types of samples have been correlated with breast neoplasia, preneoplasia, and high risk of developing breast cancer, but most of these studies were conducted in a single center with limited numbers of samples. To determine the usefulness of DNA methylation in breast cancer detection, EDRN participants at this strategic discussion recommended to systematically validate these methylation markers by an established consortium, such as the EDRN, which has the infrastructure to rapidly conduct a well-designed prevalidation or validation study. It was recommended that EDRN and non-EDRN investigators work together to conduct a specific project, using a well-defined end point, the same set of blinded specimens, appropriate experimental design and data analysis, and standard technology to evaluate methylation biomarkers to generate a reliable conclusion about the usefulness of these markers for a specific clinical use in breast cancer. Specifically, they recommended a prevalidation study in which investigators use tissues from patients with well-defined benign breast disease who developed breast cancer in 5 to 10 years and tissues from matched well patients with well-defined benign breast disease who do not develop breast cancer to determine whether methylation biomarkers or a panel of methylation markers can predict progression from breast benign diseases to malignancy.

A systematic evaluation of biomarkers must take into consideration the characteristics of different types of biomarkers. DNA methylation markers are generally not specific for breast cancer, rather, aberrant methylation of the same genes exist in several organ types of cancers. At the prevalidation stage, one direct approach is to evaluate DNA methylation markers using sample sources from breast (FNA, NAF, or DL) to determine the usefulness of the markers for breast cancer risk assessment, detection, or

stratification. In addition, it is necessary to examine the correlation of DNA gene methylation patterns between breast tissues and body fluids to develop less invasive and more convenient diagnostic assays.

During the discussion for making decision to move DNA methylation for clinical application, several key points have emerged as follows: (a) ensure that the current scientific findings in support of the proposed biomarkers are sound, reproducible, and consistent, and are supported by various laboratories; (b) perform systematic prevalidation and validation studies through using available resources that could provide suitable specimens and infrastructure, such as the EDRN, which could provide the validation resources; (c) link specific biomarkers or panel of biomarkers to specific medical use as the example of Oncotype DX discussed in the meeting; and (d) follow all required processes, regulations, and standards for the biomarkers to be commercially viable in clinical diagnosis. DNA methylation has emerged as a highly promising biomarker for future studies.

Summary

In summary, biomarkers, including DNA methylation, have shown great potential for breast cancer in personalized medicine, risk stratification, and chemoprevention response targets. However, few biomarkers or panel of biomarkers have been shown to have clinical utility, and there is no gene methylation biomarker panel for sporadic breast cancer today. To promote research on biomarkers for risk assessment and stratification, diagnosis, progression, and prevention, it is necessary to evaluate hypermethylation genes that have shown highly positive correlation with breast cancer risk to filter out those with true value and proper sensitivity and specificity for development of a panel for use in a future large-scale clinical validation study. Because most methylation biomarkers are not breast cancer specific, using breast tissue as the specimen for a methylation biomarkers prevalidation study will pinpoint breast cancer clinical issues directly. Better techniques to isolate cells derived from breast cancer in circulation may allow for the use of body fluids and less invasive or noninvasive methods to obtain specimens for DNA methylation validation and for clinical usage.

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No potential conflicts of interest were disclosed.

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