Improving early breast cancer detection: focus on methylation

K. P. M. Suijkerbuijk 1*, P. J. van Diest 1 & E. van der Wall 2

1Department of Pathology; 2Division of Internal Medicine and Dermatology, University Medical Center Utrecht, Utrecht, The Netherlands

Received 25 January 2010; revised 31 March 2010; accepted 1 April 2010

The need for additional breast cancer screening tools is indisputably high, as one may conclude from the high rate of interval malignancies in women undergoing regular screening. DNA promoter methylation frequently occurs during breast carcinogenesis and is an early event in this process. Moreover, a field defect for methylation has been described and methylation values can reliably be assessed in limited amounts of DNA. Simultaneous detection of methylation of a panel of genes in breast fluids and/or blood derivatives could be both sufficiently specific and sensitive to be of additive value to current imaging-based screening methods. This review describes the recent developments in methylation detection in breast fluids, serum and plasma that paved the way for large prospective studies. These studies will provide us with the definite answer as to what will be the additive value of defining the methylation status of specific genes to current imaging-based screening methods.

Key words: breast neoplasms, DNA methylation, early detection of cancer, nipple aspirate fluid, plasma, serum

case description

A 32-year-old woman is a known carrier of a BRCA1 germline mutation and therefore undergoes annual screening for breast cancer. Her mother died of breast cancer at the age of 36 years. She considers prophylactic mastectomy but the anticipated morbidity and physiological stress keeps her from taking the decision. Because of high breast density, mammography is not informative. Magnetic resonance imaging (MRI) previously detected aberrations that on biopsy turned out to be benign lesions, which have caused uncertainty and stress. Four months after a screening visit (physical examination and MRI), she feels a lump in the outer part of her right breast. The histology of the core biopsy shows a poorly differentiated triple-negative adenocarcinoma of the breast. A sentinel node procedure reveals metastases in the lymph nodes.

This case illustrates the limitations of current screening options for women at increased risk of breast cancer. About 7% of breast cancer cases are thought to be attributable to an inherited mechanism [1]. Carriers bear a lifetime risk up to 80% to get affected and are therefore screened at regular intervals by imaging modalities from a young age on. Especially for these young high-risk women, there is a lot to be won in terms of accurate assessment of direct breast cancer risk. High breast density in these women significantly decreases the sensitivity of mammography, resulting in a 26%–46% rate of interval malignancies [2–4]. MRI, the designated alternative, has a good sensitivity but its poor specificity leads to unnecessary breast biopsies with their associated uncertainty. Altogether, new screening methods that could improve the accuracy of detection would be of great value.

methylation

In the mammalian genome, DNA methylation takes place only at cytosine bases (C) that are located 5’ to a guanosine (G) in a CpG dinucleotide, which is significantly underrepresented [5]. Most cytosines within CpG dinucleotides are methylated in the human genome, but some remain unmethylated in short regions of 0.5–4 kb in length that are rich in CpG content, known as CpG islands [5, 6]. Over 50% of the protein-coding genes have at least one CpG island within or near their promoters. The expression of these genes can be sensitive to the methylation status of such CpG islands [7], being generally unmethylated in normal cells. In cancer, however, hypermethylation of these promoter regions within a background of global hypomethylation is now the most well-categorized epigenetic change to occur, found in virtually every type of human neoplasm [7, 8].

Methylation plays an important role in normal cells as well as in tumor development. In normal cells, it contributes to chromatin organization, silencing of transposable elements, X-chromosome inactivation, tissue-specific expression and genetic imprinting. In contrast, besides general hypomethylation of the genome [9], hypermethylation of CpG islands in gene promoter regions occurs in cancer cells [7]. This promoter hypermethylation leads to inactivation of
genes involved in cell cycle, cell adherence, DNA repair and apoptosis [7]. Obviously, this promoter hypermethylation (further denoted as ‘methylation’) process does not stand by itself but is associated with other epigenetic changes such as histone deacetylation and chromatin remodeling as reviewed by McCabe et al. [10].

Its reversible nature makes methylation an attractive therapeutic target [11, 12]. This certainly is the case for hematological malignancies, but also in solid cancers, beneficial effects have been demonstrated. Moreover, methylation has been shown to have prognostic value and predicts response to treatment [11, 12]. Above all, significant clinical benefit is to be expected of methylation as a biomarker for early detection [13], which is the focus of this review.

**methylation as a biomarker**

The importance of molecular markers in cancer detection is increasingly recognized, with the ultimate goal to detect tumors in an early preferably premalignant stage. Methylation might be just the right candidate for this purpose because of numerous favorable characteristics. First, in the process of carcinogenesis, promoter hypermethylation is a more frequently occurring event than mutations [14], with estimates varying from 600 to 1000 aberrantly methylated genes per tumor [15]. Among these are genes that are implicated in important steps in cancer development [16–18], including genes coding for microRNAs [19, 20]. Second, a field defect for methylation has been proposed, indicating that not only the malignant cells but also the surrounding tissue shows methylation defects. Yan et al. [21] demonstrated a field of epigenetic changes in the breast extending up to 4 cm from the primary tumor. This finding has important implications for ‘the intraductal approach to breast cancer’ [22]. Third, methylation has been shown to be an early event in breast tumorigenesis [23–25]. Fourth, DNA methylation is stable and can be amplified by PCR, which means that aberrations can relatively easy be analyzed in tiny amounts of material [26] as opposed to other approaches such as gene expression profiling. Lastly, a hypermethylated sequence forms a positive signal against an unmethylated background, which makes it more easily detectable than genetic alterations such as loss of heterozygosity [27].

As a consequence of this favorable profile, a multitude of methylation assays has been developed over the last decade, with different features tailored to different applications [13, 28]. The majority of DNA methylation assays are based on either sodium bisulfite (NaBi) modification of the DNA that converts unmethylated cytosines to uracil or makes use of methylation-sensitive restriction enzymes to distinguish methylated from unmethylated DNA. Table 1 lists examples of different sources of methylation analysis. The choice of the methylation assay is important. The limited amounts of DNA present in breast fluids and plasma and serum require very sensitive assays. In a recent study, we found that the commonly used NaBi-based nonquantitative methylation-specific PCR (MSP) showed a poor concordance with different quantitative NaBi and restriction enzyme-based methylation assays [29]. Quantitative multiplex methylation-specific PCR (QM-MSP) [30], an NaBi-based nested quantitative assay, has a 10-fold higher sensitivity compared with MSP, can detect methylation in <1 ng of DNA and therefore is more suitable for measuring methylation in breast fluids and serum or plasma [29]. The development of these highly sensitive PCR-based methylation assays has paved the way for methylation detection in biological fluids with low DNA content, such as sputum, urine and faeces for early cancer detection [13]. For the purpose of breast cancer detection, methylation can be detected in breast fluid or blood derivatives. While methylation detection in the lumen-derived fluids is thought to have a higher sensitivity, plasma and serum will probably contribute mainly in specificity [28]. This suggests that simultaneous methylation detection in both sources will be mandatory to ensure sufficient sensitivity and specificity to be of additive value.

As an alternative for methylation analysis, methods to detect chromatin remodeling such as chromatin immunoprecipitation and massively parallel sequencing (ChIP-seq) [31] have proven to be able to identify new genomic elements and may in the future become of value for breast cancer detection [11].

**Table 1. Various sources of methylation analysis**

<table>
<thead>
<tr>
<th>NaBi based Method</th>
<th>Restriction enzyme based Method</th>
<th>Various Method</th>
<th>Based on</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP</td>
<td>MS-MLPA</td>
<td>MIRA</td>
<td>Affinity of MBD2b for methylated DNA</td>
</tr>
<tr>
<td>QM-MSP</td>
<td>Methyl-profiler</td>
<td>COBRA</td>
<td>Combination of NaBi and restriction enzyme treatment</td>
</tr>
<tr>
<td>MethylLight</td>
<td>HpaII-PCR</td>
<td>SsOH methyl group acceptance assay</td>
<td>Radiolabeled methyl group acceptance by unmethylated DNA</td>
</tr>
<tr>
<td>Bisulfite sequencing</td>
<td>COMPARE-MS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

COBRA, combined bisulfite restriction analysis; COMPARE-MS, combination of methylated-DNA precipitation and methylation-sensitive restriction enzymes; MIRA, methylated-CpG island recovery assay; MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification; MSP, methylation-specific PCR; QM-MSP, quantitative multiplex methylation-specific PCR.
methylation detection in luminal fluids

The most straightforward way to obtain nipple fluid is nipple aspiration. Nipple aspiration is a noninvasive procedure, in which an intermittent vacuum (using a breast pump) is used to obtain nipple aspirate fluid (NAF; Figure 1). In the past, low success rates have been reported, but this problem is largely overcome by using oxytocin nasal spray [32]. We demonstrated that oxytocin-supported nipple aspiration yields sufficient DNA for methylation analysis in >90% of women, irrespective of previous treatment [32, 33]. Krassenstein et al. [34] evaluated methylation of GSTPI, RARB, p16INK4a, p14ARF, RASSF1A and DAPK in matched nipple fluid, tumor and normal tissue of 22 breast cancer patients. They found methylation of one or higher gene in 82% of nipple aspirates. Zhu et al. [35] found methylation values of CCND2, CDKN2A, RARB and RASSF1 in 18 NAFs to be in between the methylation values in cancerous and normal adjacent tissue of the same breast.

An alternative method to obtain fluid from the breast is by ductal lavage. Under local anesthesia, a catheter is inserted into the breast, after which (part of) the ductal system of the breast can be rinsed using a saline solution. Evron et al. [36] were the first to demonstrate the feasibility of methylation detection in ductal lavage fluid using MSP. Methylation of CCND2, RARB or TWIST1 was found in 11% of fluids from healthy women that showed no cytological abnormalities, in 40% of fluids with atypia and in the one fluid that contained malignant cells. Fackler et al. [37] subsequently showed that, compared with cytology, analyzing promoter methylation of a similar gene set using QM-MSP in ductal lavage cells doubled the detection rate of breast cancer, increasing sensitivity from 32% to 62%. In another study, methylation of CCND2, APC, SCGB3A1, RASSF1 and RARB in ductal lavage did not predict marked atypia but was associated with Gail model risk classifications and a personal history of breast cancer [38]. Locke et al. [39] carried out ductal lavage in healthy BRCA1/2 germline mutation carriers and controls. They found methylation of RARB, SCGB3A1, TWIST1 or CCND2 in 8 of 19 ductal fluids from the BRCA carriers (42%), while none of the 5 controls showed methylation. Antill et al. [40] recently found methylation of CDKN2A, RASSF1, TWIST or RARB in 40% of ductal fluids obtained from BRCA1 and BRCA2 germline mutation carriers.

Methylation of one or more of these genes was associated with age and a history of breast cancer. Importantly, RASSF1 methylation was associated with developing breast cancer during the study.

A more invasive way to obtain fluid from the breast is fine needle aspiration (FNA). In this procedure, either randomly or palpation-guided fluid and cells are aspirated from parts of the breast. Lewis et al. [24] carried out MSP in random periareolar FNA in breast cancer patients and unaffected women. They showed that CCND2 methylation occurred only in aspirates from breast cancer patients, while APC, RARB and RASSF1 methylation was related to predicted breast cancer risk. Another study showed that methylation of CCND2, RASSF1, APC and SCGB3A1 in a validation set of FNA washings, identified breast cancer with a 100% specificity and a 42% sensitivity when three of four genes were methylated [41]. Using palpation-directed FNA, Euhus et al. [42] showed that in breasts with benign lesions, methylation of APC and RASSF1 correlated with atypia, while methylation of CCND2 and RASSF1 and the sum of methylation was related to predicted breast cancer risk.

serum and plasma

Another approach is the detection of methylation in blood derivatives. Cell-free DNA in serum and plasma is thought to originate from necrotic and apoptotic tumor cells and can thus be used for molecular diagnosis of tumor-specific alterations such as methylation [43]. Since methylation is barely detected in serum or plasma from healthy individuals, its specificity for detecting cancer is high. Using multiple gene panels, several studies demonstrated methylation in a significant proportion of sera or plasma from breast cancer patients, while methylation was usually not detectable in serum or plasma from healthy individuals (see Table 2), as also reviewed in [44]. Generally, there was concordance between methylation in serum or plasma and tumor. Hoque et al. [45] demonstrated using a training and a test set that methylation of at least one of the genes APC, GSTPI, RASSF1 and RARB in plasma predicted breast cancer presence with a 62% sensitivity and a 87% specificity. In another study, ESR1 and 14-3-3σ methylation in serum distinguished breast cancer patients from healthy controls with an 81% sensitivity and an 88% specificity [46]. Remarkably, these methylation values did not change significantly between pre-surgery and post-treatment sera.

conclusions

Methylation of specific genes is known as an early hallmark of carcinogenesis. Today, methods to establish methylation status are definitely more refined and reliable. Since

Figure 1. Nipple aspirate fluid obtained through noninvasive aspiration with a breast pump. These small drops of fluid contain enough DNA for methylation analysis of multiple genes.
methylation can be detected in only small amount of DNA, defining the methylation status is potentially a very attractive method for early tumor detection in body fluids. Large prospective studies in high-risk women with sufficient follow-up as well as validation studies in cohorts of breast cancer patients are required to investigate the true additive value of these screening modalities to existing imaging-based screening methods.

**epilogue—breast cancer screening in 2020**

A 32-year-old woman is a known carrier of a BRCA1 germline mutation and therefore undergoes annual screening for breast cancer. Besides MRI and physical examination, nipple fluid is aspirated and blood is drawn at every screening visit and promoter methylation for a 10-gene panel is assessed. At a certain screening visit, methylation is detected in 3 of 10 genes in the nipple fluid from the right breast and in 2 genes in plasma. Results are discussed and the woman takes the decision to undergo prophylactic mastectomy. In the right breast, a small focus of high-grade ductal carcinoma in situ is found.

**funding**

Dutch Cancer Society (grant numbers UU 2007-3977, UU 2008-4217); the 'Integraal Kankercentrum Midden-Nederland'; the 'Ata Visser Stichting'; the American Women’s Club of The Hague; A Sister’s Hope/Pink Ribbon and ZonMW (AGIKO stipend).

**disclosure**

None of the authors declare conflicts of interest.

**references**


### Table 2.

<table>
<thead>
<tr>
<th>Study</th>
<th>Serum or plasma</th>
<th>Number of breast cancer cases</th>
<th>Genes</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hu et al. [47]</td>
<td>Serum</td>
<td>36</td>
<td>CDKN2A or CDH1</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Dulaimi et al. [48]</td>
<td>Serum</td>
<td>34</td>
<td>RASSF1, APC, DAPK1</td>
<td>76</td>
<td>100</td>
</tr>
<tr>
<td>Taback et al. [49]</td>
<td>Serum</td>
<td>33</td>
<td>RASSF1, RARB, MGMT, APC</td>
<td>27</td>
<td>100</td>
</tr>
<tr>
<td>Shukla et al. [50]</td>
<td>Serum</td>
<td>20</td>
<td>RASSF1</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Mirza et al. [51]</td>
<td>Serum</td>
<td>50</td>
<td>TMS1, BRCA1, ESRI or PRB</td>
<td>64</td>
<td>100</td>
</tr>
<tr>
<td>Sharma et al. [52]</td>
<td>Serum</td>
<td>36</td>
<td>CCND2, CDKN2A or SLIT2</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>Jing et al. [53]</td>
<td>Serum</td>
<td>38</td>
<td>BRCA1, CDKN2A or 14-3-3-σ</td>
<td>71</td>
<td>100</td>
</tr>
<tr>
<td>Tan et al. [54]</td>
<td>Serum</td>
<td>19</td>
<td>RUNX3, CDKN2A, RASSF1 or CDH1</td>
<td>79</td>
<td>100</td>
</tr>
<tr>
<td>Martinez-Galán et al. [46]</td>
<td>Serum</td>
<td>106</td>
<td>ESR1 or 14-3-3-σ</td>
<td>81</td>
<td>67</td>
</tr>
<tr>
<td>Van der Auwera et al. [55]</td>
<td>Serum</td>
<td>79</td>
<td>APC, RASSF1 or ESRI1</td>
<td>53</td>
<td>84</td>
</tr>
<tr>
<td>Mirza et al. [56]</td>
<td>Serum</td>
<td>100</td>
<td>14-3-3-σ</td>
<td>56</td>
<td>87</td>
</tr>
<tr>
<td>Silva et al. [57]</td>
<td>Plasma</td>
<td>35</td>
<td>CDKN2A</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td>Bae et al. [58]</td>
<td>Plasma</td>
<td>34</td>
<td>CCND2, RARB, TWIST1 or SCGB3A1</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td>Papadopoulou et al. [59]</td>
<td>Plasma</td>
<td>50</td>
<td>RASSF1 or ATM</td>
<td>36</td>
<td>100</td>
</tr>
<tr>
<td>Hoque et al. [45]</td>
<td>Plasma</td>
<td>47†</td>
<td>GSTPI, RARB, RASSF1 or APC</td>
<td>62</td>
<td>87</td>
</tr>
</tbody>
</table>

*Sensitivity is defined as percentage of serum or plasma samples from breast cancer cases that shows methylation of this gene or gene panel.

+Specificity is defined as 100% − percentage of serum or plasma samples from healthy women that shows methylation for this gene or gene panel (100% means that no methylation was detected in plasma or serum from healthy women).

+Validation set.

