Assessment of DNA methylation status in early stages of breast cancer development

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Background: Molecular pathways determining the malignant potential of premalignant breast lesions remain unknown. In this study, alterations in DNA methylation levels were monitored during benign, premalignant and malignant stages of ductal breast cancer development.

Methods: To study epigenetic events during breast cancer development, four genomic biomarkers (Methylated-IN-Tumour (MINT)17, MINT31, RARβ2 and RASSF1A) shown to represent DNA hypermethylation in tumours were selected. Laser capture microdissection was employed to isolate DNA from breast lesions, including normal breast epithelia ($n = 52$), ductal hyperplasia ($n = 23$), atypical ductal hyperplasia ($n = 31$), ductal carcinoma in situ (DCIS, $n = 95$) and AJCC stage I invasive ductal carcinoma (IDC, $n = 34$). Methylation Index (MI) for each biomarker was calculated based on methylated and unmethylated copy numbers measured by Absolute Quantitative Assessment Of Methylated Alleles (AQAMA). Trends in MI by developmental stage were analysed.

Results: Methylation levels increased significantly during the progressive stages of breast cancer development; P-values are 0.0012, 0.0003, 0.012, <0.0001 and <0.0001 for MINT17, MINT31, RARβ2, RASSF1A and combined biomarkers, respectively. In both DCIS and IDC, hypermethylation was associated with unfavourable characteristics.

Conclusion: DNA hypermethylation of selected biomarkers occurs early in breast cancer development, and may present a predictor of malignant potential.

Invasive ductal breast tumours are thought to arise from benign breast tissues through multiple transforming events over time (Wellings and Jensen, 1973; Wellings et al, 1975; Jensen et al, 1976; Allred and Mohsin, 2000). Models of breast cancer development have identified several histologically recognisable breast lesions as potentially precancerous. Ductal hyperplasia (DH), atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS) are associated with a 1.5, 5 and 10-fold increased relative risk for future invasive ductal cancer (IDC), respectively (Dupont and Page, 1985; Page et al, 1985; Dupont et al, 1993; Page et al, 1995; Hartmann et al, 2005; Degnim et al, 2007). Although such lesions signify an increased risk for the development of invasive breast cancer, not all patients will indeed acquire invasive disease. The majority of proliferative lesions does not have the molecular characteristics that enable them to progress to invasiveness. Factors determining the behaviour of proliferative lesions remain unknown. Tools for risk stratification are warranted to identify patients diagnosed with proliferative lesions of the breast who may benefit from surgical treatment or adjuvant therapy, vs patients who may safely refrain from treatment. Moreover, identification of key changes in proliferative lesions is relevant as it may render targets for novel preventative therapies. Detection and intervention during early

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stages of breast cancer development offers an opportunity to decrease the incidence of invasive breast cancer and to improve the survival of women at risk of developing breast cancer.

DNA hypermethylation is recognised as an important epigenetic aberrancy contributing to the development of several types of cancer (Esteller, 2008), including breast cancer (Lo and Sukumar, 2008). In this study, methylation levels were assessed during key steps in breast cancer development, including transitions from DH to ADH to DCIS. Four candidate methylation biomarkers known to be aberrantly hypermethylated in invasive breast cancer were selected. RARβ2 and RASSF1A are tumour suppressor genes, whose loss of expression in invasive breast tumours is known to be partially attributable to promoter hypermethylation (Sirchia et al, 2000; Agathanggelou et al, 2005; Shinozaki et al, 2005; Dumont et al, 2009; Van der Auwera et al, 2010). MINT17 and MINT31 belong to the group of Methylated-IN-Tumour (MINT) loci, and are biomarkers with known cancer-associated methylation in gastro-intestinal tumours (Toyota et al, 1999; de Maat et al, 2007), melanoma (Tanemura et al, 2009) and breast cancer (Roll et al, 2008; van Hoesel et al, 2012). This study explores the association of aberrant methylation of these four markers with malignant transformation. In DCIS and IDC, we also assessed the relation of aberrant methylation and established predictors in breast cancer, such as oestrogen receptor (ER), progesterone receptor (PR), HER2 and histological grade (Allred et al, 2009; Rakovitch et al, 2012).

Lesion size has presented an obstacle for detailed molecular studies on archival paraffin-embedded tissue specimens of proliferative breast lesions. In this study, laser capture microdissection (LCM) and in situ bisulfite modification were employed for methylation analysis by PCR, enabling assessment of small lesions (<1.5 mm). This study aimed to characterise epigenetic biomarkers associated with progression in premalignant breast lesions towards the malignant phenotype.

MATERIALS AND METHODS

Clinical Specimens. Female patients who underwent a resection biopsy of the breast and were subsequently diagnosed with ductal proliferative disease at the Department of Pathology at St. Johns Health Center between 1996 and 2008 were identified. Patients with mixed lobular and ductal lesions, or with insufficient amounts of archived formalin-fixed paraffin-embedded (FFPE) tissues were excluded. Archived FFPE tissues were obtained from a total of 235 lesions, derived from 138 patients. Selected tissues comprised normal breast tissues ($n = 52$), DH ($n = 23$), ADH ($n = 31$), DCIS ($n = 95$) and AJCC stage I IDC ($n = 34$). Ductal carcinoma in situ tissues included low-grade ($n = 30$), intermediate-grade ($n = 27$), high-grade ($n = 25$) lesions and lesions of unspecified grade ($n = 13$). The patient cohort contained both patients diagnosed

with isolated lesions, as well as patients diagnosed with (multiple) synchronous lesions. Two expert pathologists (RRT and JMS) reviewed the H&E slide for each selected tissue specimen to identify and mark proliferative lesions and normal epithelia (Figure 1).

Use of selected tissues for methylation studies was approved by the Western Institutional Review Board (Olympia, Washington).

DNA preparation from normal and (Pre)malignant breast Tissues. Laser capture microdissection was employed to harvest DNA from normal breast epithelia and proliferative ductal breast lesions. CapSure Macro LCM caps (MDS Analytical Technologies, Sunnyvale, CA, USA) were used to capture dissected tissues. Deparaffinized tissue sections of $8 \mu m$ thickness on non-adhesive glass slides were used as a tissue source. To determine the required surface area, a test was undertaken measuring copy numbers in incremental surface areas, ranging from 10^5 to $10^7 \mu m^2$. Lesion surface area for LCM capturing was determined at >1 mm^2. For each specimen, the original H&E slide marked by the pathologists served as roadmap for the accurate isolation of separate tissues of interest.

Laser capture microdissection tissues were subjected to on-cap in situ sodium bisulfite modification to enable assessment of DNA methylation levels (Sunami E et al, 2011). To denature sample DNA in preparation for sodium bisulfite modification, on-cap tissues were incubated in 0.2 M NaOH at 37 °C for 15 min. Next, captured tissues were incubated in sodium bisulfite solution at 60 °C for 8 h, rinsed with molecular grade water twice for desalting and incubated in 0.3 M NaOH at 37 °C for 15 min to finalise bisulfite modification. Bisulfite-modified DNA of on-cap tissues was brought into solution by digesting on-cap tissues in lysis buffer containing proteinase K (Sigma, St. Louis, MO, USA) overnight at 50 °C. Finally, the proteinase K enzyme was deactivated at 95 °C for 15 min.

Quantitative PCR to assess methylation levels. Methylation levels for all four genomic loci were measured by Absolute Quantitative Assessment of Methylated Alleles (AQAMA) PCR, as published previously (de Maat et al, 2007; Tanemura et al, 2009). In short, a universal set of primer was designed for each biomarker, annealing to both methylated and unmethylated bisulfite-modified DNA sequences. A pair of minor groove-binding probes was designed to specifically report amplification of methylated and unmethylated sequences. Primer and probe sequences were as follows: MINT17, 5′-AGGGGTTAGGTTGAGGTTGTT-3′ (forward), 5′-TCTACCTCTTCCCAAATTCCA-3′ (reverse), 5′-TTG GATGGATCGCGG-3′ (methylated sequence-specific (M) probe), 5′-TATTTTGGATGGATTGTGG-3′ (unmethylated sequence-specific (U) probe); MINT31, 5′-TAAAGTGAGGGGTGGTGA TG-3′ (forward), 5′-AAAAACACTTCCCCAACATCT-3′ (reverse), 5′-AGGTTTCGTCGTGTTT-3′ (M probe), 5′-AGGTTTTGTTGT GTTTAT-3′ (U probe); RASSF1A, 5′-AAGGAGGGAAGGAAGGG
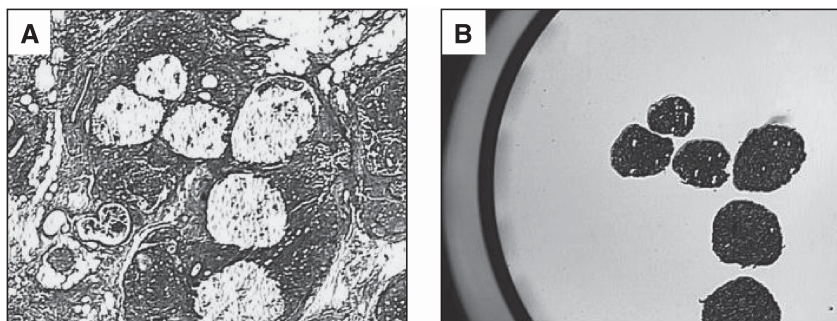
Figure 1. Laser capture microdissection of ductal carcinoma in situ. All tissue types assayed in this study were carefully microdissected by LCM from deparaffinized, hematoxylin-stained slide sections of $\sim 8 \mu m$ thickness. A representative slide example of capturing DCIS tissue is shown after LCM in (**A**), and the microdissected tissues LCM on-cap in (**B**).

TAAG-3′ (forward), 5′-CTCCCCCAAAATCCAAACTAA-3′ (reverse), 5′-TTTGCGAGAGCGCGT-3′ (M probe), 5′-TTTGTG AGAGTGTGTTTAG-3′ (U probe), and RARß2, 5′-AGTTGTTT-GAGGATTGGGATGT-3′ (forward), 5′-ATCCCAAATTCTCCTT CCAAATA-3′ (reverse), 5′-AGAACGCGAGCGATT-3′ (M probe) and 5′-AGAATGTGAGTGATTTGAG-3′ (U probe). Copy numbers of methylated and unmethylated sequences were quantified based on a separate plasmid standard curve for each. A total PCR reaction volume of $10\,\mu l$ was used, containing $2\,\mu l$ of tissue lysate, $350\,\mu mol$ of dNTP, 1.4 units Accustart DNA polymerase (QuantaBiosciences, Gaithersburg, MD, USA), 5 mmol MgCl2, $0.4\,\mu mol$ of forward and reverse primer, 0.025 pmol of each probe and ROX for passive reference fluorescence. All sample reactions were performed as triplicates. Controls included universal methylated and universal unmethylated controls as previously published (Tanemura *et al*, 2009), and zero-template controls. Performance and accuracy of the methodology used in this study has been previously demonstrated (de Maat *et al*, 2007).

Statistical analysis. Methylation levels were expressed as Methylation Index (MI), calculated from absolute copy numbers as follows: MI = methylated copy number/(methylated copy number + unmethylated copy number). The MI is a continuous value ranging from 0 to 1, and can be interpreted as methylation percentage, with MI = 0 representing complete absence of methylation and MI = 1 meaning complete methylation. Methylation Index was obtained for each sample, and mean MI was calculated for each tissue category. In addition, a standardized average MI (MI^{panel}) was calculated to assess alterations in methylation of the four biomarkers together. Both single biomarker MI and MI^{panel} were used for statistical analysis. For all statistical tests, *P*-values < 0.05 were considered significant. Pearson's correlation coefficient was used to examine the linearity between incremental LCM surface areas and DNA copy numbers for determining the required surface area. Association between MI and tumour characteristics, such as ER, PR, HER2 and grade, was examined using the χ^2 test. Trends in MI during breast cancer development were analysed using both a categorical mixed-effects model, considering the included tissue types as independent entities, and a linear mixed-effects model, considering the tissue types as related progressive steps (Beitler and Landis, 1985; Roy, 2009; Bastogne *et al*, 2010). Congruence between histological grade of DCIS and synchronous invasive carcinoma was assessed by the Fisher's exact test.

RESULTS

Linearity of LCM surface area and copy numbers. At first, a LCM test study was undertaken to determine the required tissue surface area for capturing by LCM to reliably perform the AQAMA assay, yielding approximately $> 10^2$ copy numbers upon amplification. A series of homogenous breast tissue was captured with incremental surface areas, ranging from 10^5 to $10^7\,\mu m^2$ of captured tissue. Samples were processed and used for AQAMA PCR as described above. Copy numbers were calculated for each surface area. Pearson's correlation coefficients for linearity between captured surface area and total copy numbers was 0.97 ($P = 0.002$) (Figure 2). A captured tissue surface area of $10^6\,\mu m^2$ sufficed to achieve $> 10^2$ copy numbers, and was used for the rest of the study.

Methylation levels during Breast Cancer Development. AQAMA PCR was successful in 228 (97%), 220 (94%), 225 (96%) and 199 (85%) of 235 lesions for MINT17, MINT31, RARβ2 and RASSF1A, respectively. Methylation Index of MINT17 was 0.16, 0.16, 0.24, 0.26 and 0.30 for normal breast epithelia, DH, ADH, DCIS and IDC, with increased methylation occurring in the
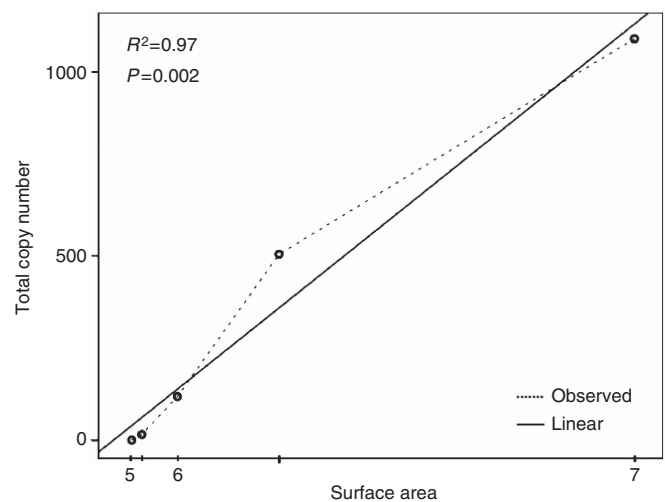

Figure 2. Linearity of LCM surface area and total copy numbers. A series of incremental surface areas of 10^5, 3×10^5, 10^6, 3×10^6 and $10^7\,\mu m^2$ was captured. Tissues were subjected to *in situ* on-cap bisulfite modification followed by proteinase K treatment. Tissue lysate was used for AQAMA PCR for copy number measurement. Data of MINT17 copy numbers are shown. The Pearson R^2 was 0.97 for correlation between increasing copy numbers with increasing surface area ($P = 0.002$). X-axis indicates the respective surface areas assayed; the numbers represent captured μm in multiples of ten. This figure also demonstrates adequate PCR efficiency for captured and processed sample DNA, yielding an efficient amplification slope (Pearson R^2 0.97) in a serial dilution series.

development from DH to ADH ($P = 0.001$ for linear trend). Likewise, RARbeta MI increased most during transition from DH to ADH, with methylation levels 0.16, 0.16, 0.29, 0.28 and 0.22 in successive tissue types (linear trend $P = 0.01$). RASSF1A MI marked the transition from normal to DH (MI 0.39, 0.75, 0.67, 0.79 and 0.76, linear trend $P < 0.001$). MINT31 showed increasing levels of methylation throughout development, but a decrease from DCIS towards IDC (MI 0.05, 0.02, 0.10, 0.21 and 0.13 for normal breast epithelia, DH, ADH, DCIS and IDC, respectively, linear trend $P = 0.003$).

Mixed-effects model analysis of MI *vs* breast cancer development stage showed a significant trend for increasing levels of methylation of all four biomarkers during progression from benign towards malignant ($P < 0.001$ for trend by both categorical and linear mixed-effects model) (Figure 3). Methylation of all four biomarkers combined (MI^{panel}) for normal breast epithelia, DH, ADH, DCIS and IDC was 0.17, 0.28, 0.33, 0.38 and 0.35, respectively.

Relationship between methylation levels and histological grade of DCIS and invasive carcinoma. Of 95 DCIS samples included in this study, 30 were low-grade, 27 were intermediate-grade and 25 were high-grade lesions. The remaining 13 DCIS samples were of unspecified histological grade. Of 34 IDCs, 10 displayed low histological grade, 12 were intermediate grade, seven were high grade and five cases were of unspecified histological grade. In 23 patients with synchronous DCIS and IDC, histological grade was known for both lesions. Histological grade of DCIS was predictive of histological grade of synchronous IDC. Congruence was present in 17 out of 23 lesions (74%) ($P < 0.001$). All six intermediate-grade DCIS concurred with intermediate-grade IDC. Of the eight high-grade DCIS, six concurred with high-grade IDC and two with intermediate-grade IDC. Five out of nine low-grade DCIS co-existed with low-grade IDC and four with intermediate-grade IDC.
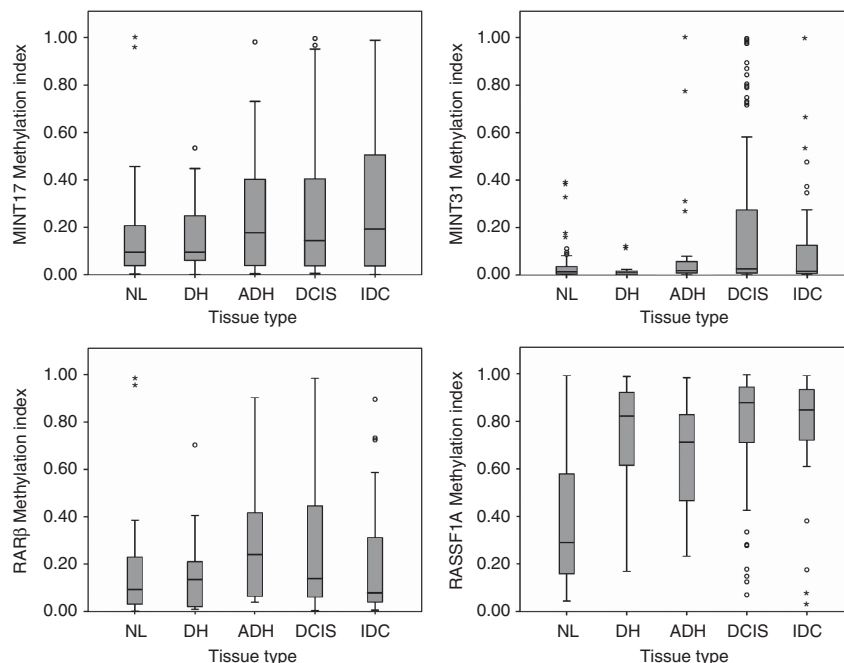
Figure 3. Methylation Index by tissue type. Boxplots showing Methylation Index of MINT17, MINT31, RARß2 and RASSF1A vs tissue type (normal breast epithelia (NL), ductal hyperplasia (DH), atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS) and invasive ductal cancer (IDC)). Boxplots show quartiles, median and outliers.

In DCIS, MI was not predictive of histological grade. Also, no significant difference was found in MI^{panel} between low-grade, intermediate-grade and high-grade DCIS. In IDC, a trend was observed towards higher MINT31 MI with higher histological grade, though not significant ($P = 0.07$).

Association between MI and ER, PR and HER2 in DCIS and Invasive Carcinoma. Oestrogen receptor, PR and HER2 were known in 28 of 34 IDC. In DCIS, ER and PR were known in 51 of 95, and HER2 was available in 29 of 95 DCIS. In DCIS, aberrant MINT31 methylation was associated with ER expression, showing increased MINT31 MI in ER-negative lesions ($P < 0.001$). Increased MI of RARβ2 was associated with HER2 amplification in DCIS ($P = 0.046$). Methylation Index^{panel} was significantly higher in ER-negative DCIS lesions ($P = 0.01$). A trend existed towards increased MI^{panel} in PR-negative DCIS ($P = 0.11$) and HER2-positive DCIS ($P = 0.10$).

In IDC, MINT31 MI was also increased in ER-negative tumours as compared with ER-positive tumours ($P = 0.046$). In addition, MINT31 MI was higher in tumours with amplification of HER2 ($P = 0.014$).

DISCUSSION

The introduction of population-wide screening mammography has caused an increase in diagnosis of invasive breast cancer. But more so, it has dramatically increased the diagnosis of clinically occult premalignant breast lesions. In 2008, ~67 770 new cases of DCIS were diagnosed (Jemal et al, 2008), compared with 4800 cases in 1983 (Ernster et al, 1996). In addition, breast biopsies prompted by abnormal mammographic findings, such as microcalcifications (Murphy and DeSchryver-Kecskemeti, 1978) or soft-tissue densities, frequently reveal benign or other premalignant lesions such as DH and ADH. This development has prompted the quest to determine clinical importance of these lesion types. It also presents the opportunity for intervention in premalignant stages. Risk assessment for women diagnosed with premalignant lesions has

become increasingly important for improved care of patients with disease of the breast.

This study aimed to identify epigenetic characteristics of premalignant lesions that may provide targets for such early intervention. So far, studies into the molecular characteristics of invasive and adjacent premalignant lesions have been undertaken analysis of genomic structural changes, loss of heterozygosity, and gene expression analysis (O'Connell et al, 1998; Buerger et al, 1999; Jeffrey and Pollack, 2003; Ma et al, 2003; Burstein et al, 2004). Fewer studies have looked into methylation aberrancies and the pattern of their occurrence during breast cancer longitudinal progression. Gene methylation governs cell differentiation, and other aberrancies which can be key changes that steer cells towards a malignant phenotype. A study indicating the importance of methylation aberrancies in early malignant transformation showed methylation of p16 to be a key factor in the earliest onset of premalignant programmes, even in normal-appearing epithelial cells (Foster et al, 1998; Crawford et al, 2004). In our study, we have taken a novel approach to illuminating methylation biomarkers in premalignant lesion. Previous reports on methylation patterns in premalignant tissues often used paired tissues, either pairing normal breast tissue with DCIS or invasive breast cancer (Hoque et al, 2009; Muggerud et al, 2010). Few studies have used a 'continuous' model as we have in our current design. Using LCM with on-cap bisulfite modification has made the study of small premalignant lesions feasible. Also, few studies so far have assessed methylation in relation to ER and HER2 status of premalignant lesion, although these are some of the most powerful predictors of invasive potential of DCIS known to date (Meijnen et al, 2008; Roses et al, 2009; Kerlikowske et al, 2010). Finally, most studies focus on individual gene coding biomarkers, whereas we used MINT markers that were previously shown indicative of genome-wide promoter hypermethylation, and as such can be regarded surrogate markers for methylation machinery malfunctioning. This study demonstrates the occurrence of methylation aberrancies during the key developmental steps of ductal breast cancer. Our data show that hypermethylation of MINT17 and RARβ2 marks the transition from proliferative epithelial hyperplasia

towards atypia, occurring especially during transition from DH to ADH. In our study, RASSF1A hypermethylation was an early marker of proliferative development, signalling the transition from normal epithelia towards DH. A study by Moelans et al (2011) suggest that DCIS is epigenetically as advanced as IDC. This is in concurrence with our results, showing aberrant methylation occurring in developmental stages as early as transition towards atypia or even proliferation. The clinical importance of hormone receptor expression in DCIS is emphasised in recent studies (Allred et al, 2012). Interestingly, we showed that aberrant methylation was associated with unfavourable lesion characteristics in DCIS with known ER, PR and HER2. Hypermethylation of MINT31 was associated with ER negativity in both DCIS and IDC. Methylation Index^{panel} was also associated with ER negativity in DCIS but not in IDC. Our previous study (van Hoesel et al, 2012) demonstrated association 'Methylation-High' or 'Meth-H' phenotype with ER positivity in IDC. As MI^{panel} and Meth-H overlap in included markers (both include MINT17, MINT31 and RARß2), these results seem contradicting. However, our current study included RASSF1A as well. Furthermore, Meth-H was defined as methylation 2 s.d.'s above normal breast tissue levels of three markers in a binary fashion, whereas MI^{panel} is a continuous value. Importantly, our current study includes AJCC stage 1 IDC only, whereas our previous study used breast tumour samples of AJCC stages 1–3. Because of these differences, results of both studies do not exclude each other. The association of aberrant methylation and hormone receptor expression in DCIS is novel and warrants exploration of precancer analysis.

Our data illustrate that aberrant methylation is an important event in the development of ductal breast cancer. Methylation markers can be useful tools to stage proliferative lesions. This study provides a first step towards the identification of methylation markers with possible value predicting malignant potential of premalignant breast lesions; it illustrates that studies assessing methylation aberrancies in association with outcome in patients with premalignant lesions are warranted. Methylation aberrancies may provide a therapeutic target in patients with premalignant lesions.

This study shows the applicability of LCM and in situ on-cap bisulfite modification for the exploration of methylation aberrancies in lesions as small as 1 mm^2, paving the way for further molecular studies dealing with very small lesions. Few studies have been published using these combined techniques. Studies previously published use pre-amplification steps to yield results, possibly introducing bias. Dietrich et al (2009) report pre-amplification while successfully retaining information on DNA methylation, thus avoiding such bias. However, the method presented in our studies makes extra amplification steps unnecessary, provided that tissue size exceeds 1 mm^2. The development of a method that saves processing steps may be valuable, as studies warranting LCM are tedious time consuming, making them less easily adaptable to clinical–pathology settings followed by small amounts of DNA extraction. The lesions size did pose a limit to the number of biomarkers that could be assessed, limiting our study to four biomarkers with techniques available. Also, our method was not suitable for very small lesions, because total tissue size needed was 1 mm^2 (for small lesions, we used multiple tissues slides).

While radiographical detection of premalignant lesions offers a great opportunity to interfere with breast cancer development at an early stage, strategies to translate this opportunity into therapy are still waiting to be developed. Importantly, not all premalignant lesions progress into cancer. To this end, tools need to be identified for risk stratification of premalignant lesions. Epigenetic aberrancies occur frequently in breast cancer, and may be candidates. This study showed that aberrant methylation of a panel of four biomarkers occurs in the early stages of breast cancer development. Targeting aberrant methylation may provide an opportunity to modulate cancer risk in the earliest stages of cancer development, at the transformation towards atypia. Striking is the big divergence in methylation levels in tissues of the same lesion type. Whether aberrant methylation in premalignant breast lesions infers a greater risk for disease progression and, eventually, the development of invasive disease, is a question that merits further research in a larger well clinically annotated cohort of patients.

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