Ductal carcinoma in situ (DCIS) is a non-obligate precursor lesion of invasive carcinoma of the breast. Current prognostic markers based on histopathological examination are unable to accurately predict which DCIS cases will progress to invasive carcinoma or recur after surgical excision. Epigenetic changes have been shown to be a significant driver of tumorigenesis, and DNA methylation of specific gene promoters provides predictive and prognostic markers in many types of cancer, including invasive breast cancer. In general, the spectrum of genes that are methylated in DCIS strongly resembles that seen in invasive ductal carcinoma. The identification of specific prognostic markers in DCIS remains elusive and awaits additional work investigating a large panel of methylatable genes by using sensitive and reproducible technologies. This review critically appraises the role of methylation in DCIS and its use as a biomarker.

DNA methylation in ductal carcinoma in situ of the breast

Jia-Min B Pang*,1,2, Alexander Dobrovic1,3 and Stephen B Fox1,3

Abstract
Ductal carcinoma in situ (DCIS) is a non-obligate precursor lesion of invasive carcinoma of the breast. Current prognostic markers based on histopathological examination are unable to accurately predict which DCIS cases will progress to invasive carcinoma or recur after surgical excision. Epigenetic changes have been shown to be a significant driver of tumorigenesis, and DNA methylation of specific gene promoters provides predictive and prognostic markers in many types of cancer, including invasive breast cancer. In general, the spectrum of genes that are methylated in DCIS strongly resembles that seen in invasive ductal carcinoma. The identification of specific prognostic markers in DCIS remains elusive and awaits additional work investigating a large panel of methylatable genes by using sensitive and reproducible technologies. This review critically appraises the role of methylation in DCIS and its use as a biomarker.

Introduction
Ductal carcinoma in situ (DCIS) is a malignant epithelial proliferation confined by myoepithelial cells and the basement membrane of breast ducts and is a non-obligate precursor to invasive carcinoma of the breast. The reported incidence of DCIS, once a rare diagnosis, has increased since the introduction of mammographic screening programs and has been reported to account for approximately 25% of new diagnoses of breast cancer [1].

The aim of DCIS treatment is to prevent progression to invasive carcinoma and subsequent potential for metastatic disease and death. DCIS is treated primarily by surgical excision, which can be in the form of breast-conserving surgery (lumpectomy) or mastectomy. Although mastectomy is considered to be curative, the recurrence rate in patients with DCIS treated with breast-conserving surgery alone has been reported to be greater than 25% over 10 years [2]. As a result, patients treated with breast-conserving surgery may also receive radiotherapy and hormonal therapy. Although several large clinical trials have reported a significant reduction in recurrence rates with the addition of adjuvant treatments in patients treated with breast-conserving surgery for DCIS [2-8], such treatments are associated with significant financial cost and side effects [9]. Since nearly 75% of DCIS cases do not recur after surgical excision [2], there is a group of low-risk DCIS patients who would not gain additional benefit from adjuvant treatment [10]. Accurately identifying this group of patients is desirable, not only to avoid side effects of treatment but also to allow better allocation of limited health resources.

Current prognostic markers in DCIS
The currently known prognostic markers of DCIS were comprehensively reviewed recently by Wang and colleagues [11] and Lari and Kuerer [12]. Known adverse prognostic factors include young age [2,3], symptomatic detection [11], and multifocal disease [11,13]. Histopathological features, such as large tumor size [11,14], high nuclear grade [11,13], the presence of comedo necrosis [11,15], positive excision margin status [11,16], negative hormone receptor status [11,12], and HER-2 amplification [11,14,17-19], have also been associated with increased risk of recurrence. Immunohistochemical detection of a range of biomarkers, including COX2 [20,21], Ki67 [20,21], p16 [20-22], p53 [18,23], p21 [17], and BNIP3 [24] as individual markers or in combination, has been associated with disease recurrence risk. Gene expression profiling has also been reported to be useful in identifying tumors with increased risk of recurrence [25-27].

Unfortunately, traditional prognostic markers are not adequate to identify low-risk DCIS patients who may be spared adjuvant hormonal treatment, and currently there is a lack of strong level I or II evidence supporting the omission of adjuvant radiotherapy in selected low-risk cases [28]. Thus, novel biomarkers are urgently required to improve individual risk-profiling and aid treatment selection. DNA methylation of a selected panel of genes
represents another potential set of markers for outcome prediction that are less dependent on scrupulous handling of the biopsy after resection [29] and remain stable even in formalin-fixed, paraffin-embedded material.

Assessing DNA methylation

DNA methylation is the addition of a methyl group at the carbon 5 position of cytosine by the action of DNA methyltransferase enzymes. In mammalian cells, cytosine methylation occurs predominantly at CpG dinucleotides. Regions of high CpG dinucleotide density, known as CpG islands, frequently exist in the promoter region of human genes. Aberrant hypermethylation of these promoter CpG islands can result in functional silencing of genes by the recruitment of histone deacetylases, resulting in the formation of inactive chromatin. Alterations of DNA methylation patterns are near universal in cancer. In particular, inactivation of tumor suppressor genes by promoter hypermethylation can be a driver of tumorigenesis.

Commonly used methodologies for DNA methylation analysis have been comprehensively reviewed elsewhere [29-32] and are summarized in Table 1. As all methodologies have their advantages and limitations, interpretation of methylation results requires critical consideration of the methodology used. It should be noted that some commonly used methodologies are prone to artifacts, in particular methylation-specific polymerase chain reaction (MSP) approaches, which require stringent primer hybridization conditions to minimize false-positive amplification. Methods that depend on restriction enzyme digestion also may give rise to false positives if digestion is incomplete.

In general, non-quantitative methods of methylation analysis should be avoided as they detect only the presence or absence of methylation regardless of the extent of methylation and will score a sample as methylated even if only a small proportion of templates are methylated. Low-level methylation means that only a small proportion of the cells being analyzed (possibly not related to the tumor) are methylated and this may not result in detectable changes in gene transcription overall. Quantitative or semi-quantitative methodologies are required to differentiate low-level from high-level methylation. These include MethyLight and similar quantitative MSP technologies, DNA sequencing, and methylation-sensitive high-resolution melting (MS-HRM). It should also be noted that when methylation is heterogeneous (that is, the individual CpGs within a given region show variable methylation), even quantitative methodologies can give variable results [30].

The choice of the appropriate region to be analyzed is also a source of variation and can lead to major discrepancies in results between studies. In general, the best region to use for most studies is the one where DNA methylation is most closely correlated to the transcription of the gene, although this is rarely assessed.

DNA methylation in DCIS

Over the last decade, relatively few studies have specifically investigated DNA methylation in DCIS. Of these, most have taken a candidate gene approach, investigating genes known to be methylated or silenced in invasive breast cancers, breast cancer cell lines, or other cancer types [33-49]. From such studies, aberrant methylation has been reported in a large variety of genes, including every pathway involved in carcinogenesis. An additional table lists these genes and their reported methylation frequencies (Additional file 1). Widely divergent frequencies of methylation have been reported for some genes, reflecting not only differences in patient groups but also the use of different non-standardized methodologies [29].

As with many biomarker studies in DCIS, studies examining DNA methylation have generally included only a relatively small number of cases. For methylation, the issue is further compounded since optimally only the neoplastic element should be assessed and this requires macro-dissection or micro-dissection to isolate DCIS from the surrounding tissues so as to avoid contamination. The amount of DNA obtainable from DCIS lesions, which are commonly small in mass, is therefore often a limiting factor in the number of cases able to be included in studies. Furthermore, not all studies have used pure DCIS cases (cases of DCIS without associated invasive carcinoma) or have combined methylation results of DCIS occurring in the context of invasive ductal carcinoma (IDC) mixed with pure DCIS cases. Studying pure DCIS cases may be critical for several reasons. The in situ component of mixed DCIS-IDC has been reported to be genomically similar to the invasive component [50], whereas DCIS-IDC combined and pure DCIS have been reported to be genetically distinct [51]. In addition, a lesion that morphologically resembles DCIS may be the spread of invasive carcinoma along a duct and therefore would be expected to have the same genetic and epigenetic alterations as invasive carcinoma.

Summary of main published studies

The published studies (Additional file 1) illustrate the complexity of assessing the overall picture of DNA methylation in DCIS. These studies have investigated different sets of genes, used different methodologies, and examined different regions of the promoter. Whereas most studies have assessed methylation as either present or absent, some have reported methylation levels as a continuous variable. These then employ various cutoffs to determine the frequency and correlation of aberrant methylation with clinicopathological parameters.
| Method                          | Brief outline of method                                                                 | Advantages                                                                 | Disadvantages                                                                 | Detection limit | Reference          |
|--------------------------------|----------------------------------------------------------------------------------------|---------------------------------------------------------------------------|-------------------------------------------------------------------------------|----------------|-------------------|
| Direct bisulfite sequencing    | Sanger sequencing of bisulfite-modified DNA sequences                                    | Allows semi-quantitative detection of DNA methylation as an average for each CpG position. Possible to sequence longer sequence lengths compared with pyrosequencing. | Difficulty in obtaining clean reads at start of sequence. Heterogeneous methylation associated with poor peak quality in four-dye electropherograms and underestimation of total DNA methylation. | 10%-20% variant base pairs | Frommer et al [80] (1992), Clark et al [81] (1994). |
| Bisulfite pyrosequencing       | Sequencing by synthesis technique that allows quantification of methylation at individual CpG positions | Quantitative method. Clean reads at beginning of sequence.               | Able to sequence relatively short sequence lengths of about 80 bp. Can identify but not quantify heterogeneous methylation. | 10% at each CpG position | Colella et al [82] (2003), Tost et al [83] (2003). |
| Mass spectrometry              | Bisulfite-modified DNA amplified using methylation-independent primers followed by base-specific cleavage of nucleic acids. Methylated and unmethylated fragments differ in mass and separated by mass spectrometry. | Detects both methylated and unmethylated sequences. Quantitative method which gives an average reading for each CpG site or region. Possible to analyze longer sequence lengths compared with pyrosequencing. | Interrogation of individual CpG sites not always possible with fragments which contain several CpG sites. | 5% at each CpG position | Ehrich et al [84] (2003), Coolen et al [83] (2007). |
| Methylation-specific PCR (MSP) | Methylation-specific primers amplify methylated bisulfite-modified DNA                   | Very high sensitivity                                                    | Detects methylated sequences only. False-positive results may occur from poor primer design, amplification of a minor methylated subpopulation, and from incomplete bisulfite modification. Non-quantitative. | 0.001%          | Herman et al [86] (1996). |
| MethylLight                    | MSP combined with Taqman probe to allow quantification of amplification in real time     | Allows quantification of DNA methylation in homogeneously methylated samples. Reduced false positives due to incomplete bisulfite conversion compared with MSP. | Detects methylated sequences only. Reduced sensitivity with heterogeneous methylation and only semi-quantitative at best in context of heterogeneous methylation. | <0.001%         | Eads et al [87] (2003). |
| Sensitive melting analysis after real-time methylation-specific PCR (SMART-MSP) | Methylation-specific primers amplify methylated bisulfite-modified DNA with quantification of amplification by use of fluorescent dye. PCR amplification followed by melt step which allows detection of false-positive results. | Quantitative for homogenous methylation. Melt step allows detection of false positives. | Detects methylated sequences only. Cannot quantify heterogeneous methylation. | 0.10%           | Kristensen et al [88] (2008). |
| Methylation-sensitive high-resolution melting (MS-HRM) | Methylation-independent primers amplify bisulfite-modified DNA sequences. Methylated and unmethylated sequences differentiated based on differing melting profiles. Process can be monitored in real time by use of a fully saturating double-stranded DNA-binding dye and can be semi-quantitative by comparing the melting profile of the sample with controls of known methylation levels. | Semi-quantitative. Detects both methylated and unmethylated sequences | Can detect presence of, but not quantify, heterogeneous methylation | 0.1%-1.0%       | Wojdacz and Dobrovic [89] (2007). |
| Methylation-sensitive single-strand conformation analysis (MS-SSCA) | Bisulfite-modified DNA amplified by using methylation-independent primers. Methylated and unmethylated amplicons form different conformers and separated by electrophoresis. | Detects both methylated and unmethylated sequences | Non-quantitative method. False positives and negatives can occur from co-migration of conformers. | 5%              | Bianco et al [90] (1999). |
Table 1. Continued

| Method                                           | Brief outline of method                                                                 | Advantages                                                                 | Disadvantages                                                                                           | Detection limit | Reference                                                                                           |
|--------------------------------------------------|----------------------------------------------------------------------------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------|-----------------|-----------------------------------------------------------------------------------------------------|
| Methylation-sensitive single-nucleotide primer extension (MS-SNuPE) | Nucleotide incorporated to extend primer placed immediately adjacent to C of CpG used to calculate average methylation at a given CpG position. | Quantitative method. Detects both methylated and unmethylated sequences. | Investigates only one CpG site with each primer. Limited ability to place primers in regions of high CpG density. |                 | Gonzalgo and Jones [91] (1997), Gonzalgo and Jones [92] (2002)                                      |
| Combined bisulfite restriction analysis (COBRA)   | Amplified bisulfite-modified DNA is digested with restriction endonucleases and the fragments separated by gel electrophoresis. | Detects both methylated and unmethylated sequences                        | Analysis possible for up to 2 CpG sites only due to each restriction endonuclease having limited number of cutting sites. Reduced cutting efficiency of restriction endonuclease leads to underestimation of DNA methylation level. Heterogeneous methylation results in underestimation of methylation levels due to formation of heterodimers. | 1%              | Xiong and Laird [93] (1997)                                                                        |
| Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) | Probe hybridized to denature DNA. Probe-DNA complex simultaneously ligated and digested by methylation-specific endonucleases. Complexes with a methylated target CpG site will not be digested, resulting in an amplification product. Complexes with an unmethylated target CpG site are digested and no amplification product results. | Semi-quantitative technique able to analyze multiple sites simultaneously. Avoids bisulfite modification, and can be used for single-stranded, short (50-60 bp) DNA sequences. | Methylation analysis restricted to methylation-sensitive restriction sites and dependent on enzyme efficiency. Fixation can reduce enzyme cleavage efficiency resulting in false positives. |                 | Nygren et al. [94] (2005)                                                                          |
| Digital techniques                               | Techniques which use limiting dilution to allow analysis of single template epialleles. | Avoids potential PCR amplification bias. Accurate quantification of heterogeneous methylation. | Requires appropriate instrumentation                                                                    |                 | Candiloro et al. [95] (2008), Candiloro and Dobrovic [96] (2009), Li et al. [97] (2009), Mikeska et al. [30] (2010) |

bp, base pairs; PCR, polymerase chain reaction.
Earlier studies specifically addressing DNA methylation in DCIS, or methylation changes in the progression to invasive carcinoma, examined mostly a single gene [33-35, 38-42,52] or at most a handful of genes [36,37]. More recently, studies have investigated panels of selected genes [46-49,53], and a small number of studies have incorporated global approaches for methylation analysis [54,55].

Several studies have shown an increase in the number of methylated genes from normal breast tissue to benign lesions to in situ carcinoma [47,49,56]. However, for most genes, methylation has been reported to occur with similar frequency in DCIS as in IDC [47,49]. This suggests that, in most cases, aberrant methylation occurs before the acquisition of an invasive phenotype and may not contribute to the development of invasion. Nevertheless, a small number of genes such as APC, CACNA1A, CDH1, FOXC1, HOXA10, MGMT, SFPR1, TFAP2A, and TWIST1 have been reported to show differences in either frequency or density of methylation between DCIS and invasive carcinoma (Table 2). This raises the possibility of using quantitative methylation of a panel of such genes to predict disease progression.

For example, Fackler and colleagues [37] reported TWIST1 methylation, as detected by MSP, to occur more frequently in IDC (15/27, 56%) compared with grade 3 DCIS (7/18, 39%), grade 2 DCIS (3/12, 25%), and grade 1 DCIS (2/14, 14%), and the difference in methylation frequency between IDC and combined grade 1 and 2 DCIS was statistically significant (P = 0.01). Douglas and colleagues [40] found methylation of TFAP2a, as detected by nested MSP, to be much more frequent in IDC (12/16, 75%) compared with DCIS (3/19, 16%, P < 0.001), although one would query whether such a sensitive methodology would yield biologically sensible results.

More recently, Hoque and colleagues [46] examined the methylation status of nine genes in pure DCIS lesions and mixed DCIS-IDC lesions by using quantitative methylation-specific polymerase chain reaction (qMSP) and chose the cutoff for aberrant methylation on the basis of receiver operating characteristic (ROC) curves. In mixed DCIS-IDC tumors, trends to higher frequencies of APC and CDH1 methylation were found in IDC compared with DCIS. APC methylation was found in 15 (38%) of 40 DCIS samples and in 24 (53%) of 45 IDC samples, and CDH1 methylation was present in 12 (31%) of 40 of DCIS samples and 21 (47%) of 45 of invasive samples.

Muggerud and colleagues [47] examined promoter methylation in pure DCIS lesions, mixed DCIS-IDC lesions, early-stage IDCs, and normal breast tissue in order to identify potential markers of DCIS progression. The analysis was done quantitatively by bisulfite pyrosequencing, and aberrant hypermethylation was defined as methylation levels two times above the standard deviation of the average of the normal controls. This study identified ABCB1, FOXC1, PPP2R2B, and PTEN as recurrently methylated genes in DCIS: all had been previously reported in IDC. Methylation of FOXC1 was observed to occur with greater frequency in invasive tumors (15/28, 53.6%) compared with pure DCIS (6/27, 22.2%).

An interesting finding of the above study [47] was reduced FOXC1 gene expression (as detected by quantitative reverse transcription-polymerase chain reaction, or qRT-PCR) relative to normal breast tissue not only when the tumor tissue was methylated but also in those tumors that were unmethylated. This echoes numerous other studies in which methylation of a given gene promoter is seen in a subset of tumors that are more generally silenced for that gene. Mechanisms other than methylation, especially histone modifications, are known to result in gene silencing, and it has been shown that gene silencing may precede DNA methylation and thus these tumors may show varying stages along the route from histone-based silencing to histone and methylation-based silencing [57].

Park and colleagues [49] investigated cases of pure DCIS, IDC, the non-malignant epithelial lesions atypical ductal hyperplasia and flat epithelial atypia, and normal breast tissue for methylation of 15 genes by MethylLight. This study reported several novel methylated genes in DCIS (DLEC1, GRIN2B, HOXA1, MT1G, SFPR4, and TMEFF2). Although methylated genes accumulating at each step of abnormality were identified, no differences in methylation frequencies between DCIS and IDC were found for most genes, with the exception of HOXA10, which was more frequently methylated in IDC (17/50, 34%) compared with DCIS (3/35, 9%) (P = 0.007).

Moelans and colleagues [48] investigated promoter methylation of 25 genes in mixed DCIS-IDC cases with methylation-specific multiplex-dependent probe amplification (MS-MLPA). No differences in the number of methylated genes between the DCIS and invasive components were observed. Verschuur-Maes and colleagues [53] also used MS-MLPA to analyze promoter methylation of a panel of 50 genes in 15 columnar cell lesions (a benign epithelial lesion) and co-existent DCIS (n = 12) and IDC (n = 14). Whereas the number of methylated genes differed between normal breast tissue and lesonal tissue, no statistical difference in the number of methylated genes was found between columnar cell lesions, DCIS, and IDC. However, MGMT and CACNA1A individually were observed to be more frequently methylated in invasive cancer compared with DCIS (MGMT methylation: 8/14 invasive, 2/12 DCIS, P = 0.022; CACNA1A methylation: 6/14 invasive, 1/12 DCIS, P = 0.048). Controversially, both studies [48,53] using...
MS-MLPA reported methylation of \textit{BRCA2} in a sizeable number of tumors, and this casts doubt on the specificity of this methodology. In some studies, methylation levels of genes have been assessed by using quantitative real-time polymerase chain reaction (qPCR). These show that methylation levels are higher in IDC than DCIS. How much this reflects tumor heterogeneity and how much this represents methodological issues in assessing increased densities of methylation remain uncertain. Significantly higher \textit{SFRP1} methylation levels have been observed in IDC compared with DCIS [49]. Similarly, Muggerud and colleagues [47] found significantly higher \textit{FOXC1} methylation levels in IDC (\(P = 0.007\)) and mixed tumors (\(P < 0.004\)) compared with DCIS-IDC, \(P < 0.04\). Higher methylation levels of \textit{MGMT} (\(P = 0.019\)) [53] and \textit{CDH1} (\(P < 0.04\)) [46] in IDC compared with DCIS have also been reported.

Whereas the above studies examined gene-specific DNA methylation at stages of breast cancer progression, Lee and colleagues [44] examined differences in DNA methylation in DCIS between American and Korean women. Quantitative multiplex methylation-specific polymerase chain reaction (QM-MSP) was used to assess methylation of a panel of 10 genes in DCIS lesions from 52 American and 48 Korean women and normal breast tissue. Although Korean women have a markedly lower incidence of DCIS, the patterns of methylation were similar in the two groups, indicating that similar mechanisms of pathogenesis underlie DCIS in the two populations.

### Global methylation studies of DCIS

Recently, studies have taken a global methylation approach to investigating DNA methylation in DCIS. Tommasi and colleagues [55] identified 108 aberrantly methylated CpG islands by methylated CpG island recovery assay-assisted microarray analysis (MIRA) in early-stage breast cancer and six cases of undissected DCIS. Candidate genes were identified on the basis of these methylated CpG islands, and six novel aberrantly methylated genes in DCIS (\textit{TLX1}, \textit{HOXB13}, \textit{HNF1B}, \textit{GFI1}, \textit{NR2E1}, and \textit{HLXB9}) were verified by combined bisulfite restriction analysis (COBRA). However, though identified as novel, these were not validated in an independent cohort of DCIS to assess their significance or used to examine the issue of recurrence or progression.

Another recent study used a global methylation approach to identify methylated genes on a panel of low-grade invasive breast cancer and \textit{in situ} cancer and then

| Gene   | Type of difference | DCIS versus IDC | Reference |
|--------|--------------------|-----------------|-----------|
| APC    | Frequency of methylation | IDC 24/45 (53%) | Hoque et al. [46] (2009) |
|        |                    | Mixed DCIS-IDC 15/40 (38%) |           |
| CACNA1A| Frequency of methylation | IDC 6/14 (42.9%) | Verschuur-Maes et al. [53] (2012) |
|        |                    | DCIS 1/12 (8.3%) |           |
|        |                    | \(P = 0.048\) |           |
| CDH1   | Frequency of methylation | IDC 21/45 (47%) | Hoque et al. [46] (2009) |
|        | Level of methylation | Mixed DCIS-IDC 12/40 (31%) |           |
|        |                    | Higher methylation levels in IDC compared with mixed DCIS-IDC, \(P < 0.04\) |           |
| FOXC1  | Level of methylation | Higher methylation levels in IDC compared with DCIS | Muggerud et al. [47] (2010) |
|        |                    | IDC versus pure DCIS \(P = 0.007\) |           |
|        |                    | IDC versus mixed DCIS-IDC \(P = 0.001\) |           |
| HOXA10 | Frequency of methylation | IDC 17/50 (34%) | Park et al. [49] (2011) |
|        |                    | DCIS 3/35 (9%) |           |
|        |                    | \(P = 0.007\) |           |
| MGMT   | Frequency of methylation | IDC 8/14 (57.1%) | Verschuur-Maes et al. [53] (2012) |
|        | Level of methylation | DCIS 2/12 (16.7%) |           |
|        |                    | \(P = 0.022\) |           |
|        |                    | Higher methylation levels in IDC compared with DCIS, \(P = 0.019\) |           |
| SFRP1  | Level of methylation | Higher methylation levels in IDC compared with DCIS, \(P = 0.035\) | Park et al. [49] (2011) |
| TFAP2A | Frequency of methylation | IDC 12/16 (75%), | Douglas et al. [40] (2004) |
|        |                    | DCIS 3/19 (16%), \(P < 0.001\) |           |
| TWIST1 | Frequency of methylation | IDC 15/27 (56%), | Fackler et al. [37] (2003) |
|        |                    | Grade 1-2 DCIS 5/26 (19%) |           |
|        |                    | \(P = 0.01\) |           |

DCIS, ductal carcinoma \textit{in situ}; IDC, infiltrating ductal carcinoma.
Table 3. Relationship between methylated genes and previously reported prognostic and predictive factors in ductal carcinoma in situ

| Parameter          | Methylated gene | Relationship                                         | Data                                | Reference                        |
|--------------------|-----------------|------------------------------------------------------|-------------------------------------|----------------------------------|
| Nuclear grade      | APC              | Higher methylation frequency in high-grade DCIS compared with low/intermediate-grade DCIS | Low/intermediate-grade DCIS 15% High-grade DCIS 60% P < 0.006 | Park et al. [49] (2011)          |
|                    | CCND2            | Higher levels of methylation with increasing Van Nuys grade | P < 0.001                           | Lehmann et al. [36] (2002)       |
|                    | CDH1             | Increased methylation frequency with increasing nuclear grade in Korean patients | Low-grade DCIS 0% Intermediate-grade DCIS 27% High-grade DCIS 44% P < 0.05 | Lee et al. [44] (2008)           |
|                    | CDKN2A           | Higher levels of methylation with increasing nuclear grade, although still low-level methylation (<10%) | Low-grade DCIS 1% Intermediate-grade DCIS 4% High-grade DCIS 7% P < 0.002 | Moelans et al. [48] (2011)       |
|                    | GSTP1            | Higher levels of methylation with increasing nuclear grade | Low-grade DCIS 6% Intermediate-grade DCIS 26% High-grade DCIS 28% P < 0.002 | Moelans et al. [48] (2011)       |
|                    | RARB             | Higher methylation frequency in high-grade DCIS compared with low/intermediate-grade DCIS | Low/intermediate-grade DCIS 15% High-grade DCIS 53% P = 0.027 | Park et al. [49] (2011)          |
| ER status          | ABCB1            | Higher levels of methylation in ER-positive tumors | Combined results of pure DCIS, mixed DCIS, and IDC cases, P = 0.003 | Muggerud et al. [47] (2010)      |
|                    | FOXC1            | Higher levels of methylation in ER-positive tumors | Combined results of pure DCIS, mixed DCIS, and IDC cases, P = 0.009 | Muggerud et al. [47] (2010)      |
|                    | GSTP1            | Higher levels of methylation in ER-positive tumors | Combined results of pure DCIS, mixed DCIS, and IDC cases, P = 0.003 | Muggerud et al. [47] (2010)      |
|                    | RASSF1A          | Higher levels of methylation in ER-positive tumors | Combined results of pure DCIS, mixed DCIS, and IDC cases, P = 0.003 | Muggerud et al. [47] (2010)      |
| PR status          | GSTP1            | Higher levels of methylation in PR-positive tumors | Combined results of pure DCIS, mixed DCIS, and IDC cases, P = 0.009 | Muggerud et al. [47] (2010)      |
| HER2 amplification | DLEC1            | Higher frequency of methylation in HER2-amplified DCIS | Non-HER2-amplified 26% HER2-amplified 75% P = 0.032 | Park et al. [49] (2011)          |
| Ki67 index         | ABCB1            | Higher methylation levels in tumors with Ki67 < 10% | Combined results of pure DCIS, mixed DCIS, and IDC cases, P = 0.006 | Muggerud et al. [47] (2010)      |
| TPS3 mutation status | ABCB1           | Higher levels of methylation in TPS3 wild-type tumors | Combined results of pure DCIS, mixed DCIS, and IDC cases, P = 0.015 | Muggerud et al. [47] (2010)      |
|                    | FOXC1            | Higher levels of methylation in TPS3 wild-type tumors | Combined results of pure DCIS, mixed DCIS, and IDC cases, P = 0.006 | Muggerud et al. [47] (2010)      |
|                    | PPP2R2B          | Higher levels of methylation in TPS3 wild-type tumors | Combined results of pure DCIS, mixed DCIS, and IDC cases, P = 0.025 | Muggerud et al. [47] (2010)      |
|                    | PTEN             | Higher levels of methylation in TPS3 wild-type tumors | Combined results of pure DCIS, mixed DCIS, and IDC cases, P = 0.01 | Muggerud et al. [47] (2010)      |

DCIS, ductal carcinoma in situ; ER, estrogen receptor; IDC, infiltrating ductal carcinoma; PR, progesterone receptor.

Profiled selected novel genes against a small number of additional in situ and invasive breast cancers [54]. The hypermethylated regions, identified by methyl-CpG immunoprecipitation and human CpG island arrays, were used to select candidate genes on the basis of the extent and frequency of methylation changes and the proximity of these changes to the gene promoters. Methylation of these selected genes was then analyzed by
mass spectrometry on a validation set that included seven DCIS cases. Eleven genes (BCAN, HOXD1, KCTD8, KLF11, NXPH1, PCDH10, POU4F1, RYR2, SIM1, TAC1, and TCF7L1) were validated as being aberrantly methylated in DCIS and IDC compared with normal breast tissue. However, methylation levels of these 11 genes in DCIS were not statistically different compared with invasive tumors for any of the genes.

**DNA methylation as a predictive and prognostic marker in DCIS**

In invasive breast carcinoma, the methylation status of certain genes has been reported to be associated with survival [58-62], risk of metastatic disease [63-66], risk of disease recurrence [62,64], and response to adjuvant treatment [60,67,68]. However, in DCIS, no direct link between aberrant methylation and risk of recurrence, risk of progression to invasive disease, or likelihood of response to adjuvant therapy has been reported.

However, previous reports indicate associations between certain methylated genes and known predictive factors such as hormone receptor status and HER2 amplification and adverse prognostic markers such as high nuclear grade, high proliferation index, TP53 mutations, and HER2 amplification [36,44,47-49] (Table 3). Although associations between methylated genes and currently known prognostic and predictive factors suggest that DNA methylation may have a role as a biomarker in DCIS, it has to be noted that these associations are the results of single studies that had relatively small numbers and that used different methylation analysis methodologies. Importantly, these studies were not designed to investigate the relationship of methylation with clinical outcome. Further well-powered studies on larger gene sets with detailed clinical data are required to establish the role of DNA methylation as a prognostic and predictive marker in DCIS.

**Future outlook**

There are inherent difficulties in conducting methylation studies in DCIS and this is due in large part to the nature of the disease itself. Pure DCIS cases are relatively rare compared with DCIS occurring in the context of invasive carcinoma, fresh tissue is almost never available, and usually only small amounts of formalin-fixed, paraffin-embedded tissue are available as a source of DNA. In addition, the use of robust methodologies for DNA methylation analysis is essential for the appropriate interpretation of methylation status.

Current knowledge of DNA methylation in DCIS is based largely on studies employing a candidate gene approach to methylation analysis. Global approaches, involving either high-throughput microarray-based assays such as the Infinium platform or one of a variety of approaches using the power of massive parallel sequencing (MPS), are now required.

The application of MPS platforms in genome-wide methylation analysis and their relative advantages and disadvantages have been comprehensively reviewed by several authors [69-73]. MPS has already been used for genome-wide methylation analysis of non-small cell lung cancer tissue [74], colon cancer tissue [75], prostate cancer cell lines and tissue [76], and breast cancer cell lines [77-79].

In addition to the identification of aberrant DNA methylation in the DCIS genome, the effect of methylation on gene expression and, importantly, clinical outcomes needs to be addressed. As DCIS is a heterogeneous disease with relatively few disease events occurring over decades, studies involving large numbers of pure DCIS cases with detailed clinical annotation and long-term follow-up are required to establish the validity of aberrant methylation as a predictive and prognostic biomarker in DCIS.

**Conclusions**

Identifying patients in whom DCIS will recur or progress to invasive carcinoma after surgical excision would allow appropriate allocation of limited health resources and avoid overtreatment of patients at low risk of further disease. DNA methylation has been found to be a predictive and prognostic marker in many forms of cancer. Although studies have shown that DNA methylation exists and may play a role in determining outcome in DCIS, we currently have an incomplete understanding of the role of DNA methylation in this disease. Studies specifically designed to investigate the relationship between DNA methylation and clinical outcome in DCIS are required to establish the validity of aberrant DNA methylation as a predictive and prognostic biomarker in DCIS.

**Additional file**

Additional File 1. Summary of genes reported to be methylated in ductal carcinoma in situ.

**Abbreviations**

DCIS, ductal carcinoma in situ; IDC, infiltrating ductal carcinoma; MPS, massive parallel sequencing; MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification; MSP, methylation-specific polymerase chain reaction.

**Competing interests**

The authors declare that they have no competing interests.

**Acknowledgments**

This work was supported by funding to SF from Cancer Australia and to AD from the National Breast Cancer Foundation. We thank Ida Candiloro, Jonathan Weiss, Stephen Wong, and Elena Takano for critically reviewing the manuscript.
References

1. Virmg BA, Tuttle TM, Shamliyan T, Kane RL. Ductal carcinoma in situ of the breast: a systematic review of incidence, treatment, and outcomes. J Natl Cancer Inst 2010, 102:170-178.

2. EORTC Breast Cancer Cooperative Group; EORTC Radiotherapy Group, Bijker N, Meijnen P, Peterse JL, Boogaerts J, van Hoevebeeck I, Julien JP, Gennaro M, Rouanet P, Avril N, Fentiman IS, Bartelink H, Rutgers EJ. Breast-conserving treatment with or without radiotherapy in ductal carcinoma-in-situ: ten-year results of European Organisation for Research and Treatment of Cancer randomized phase III trial 10853-a study by the EORTC Breast Cancer Cooperative Group and EORTC Radiotherapy Group. J Clin Oncol 2006, 24:3381-3387.

3. Fisher B, Dignam J, Wolmark N, Wickerham DL, Fisher ER, Mamounas E, Smith TL, Wolmark N, Wickerham DL, Fisher ER, Mamounas E, Smith TL: EORTC Breast Cancer Cooperative Group and EORTC Radiotherapy Group. J Clin Oncol 2006, 24:3381-3387.

4. Omlin A, Amichetti M, Azria D, Cole BF, Fourneret P, Poortmans P, Naehrig D, Fisher B, Dignam J, Wolmark N, Wickerham DL, Fisher ER, Mamounas E, Smith TL: EORTC Breast Cancer Cooperative Group and EORTC Radiotherapy Group. J Clin Oncol 2006, 24:3381-3387.

5. Ganz PA, Trudeau ME, Coldman AJ, Chi DS, Buzdar AU, Huang SC, Sheiman RG, Hortobagyi GN: Breast carcinoma in situ: recent advances and future prospects. Int J Radiat Oncol Biol Phys 2002, 53:21-29.

6. Pairolero PC,egotter CD, Skandalakis JE. Ductal carcinoma in situ of the breast with excision alone. Cancer 2001, 91:1547-1554.

7. Mehta KJ, Sporn MB: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

8. Palekar AR, Varambally S, O’Malley AK, Bhatia S, Jacks T, Bova GS: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

9. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

10. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

11. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

12. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

13. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

14. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

15. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

16. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

17. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

18. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

19. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

20. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

21. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

22. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

23. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

24. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

25. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

26. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

27. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

28. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

29. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

30. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

31. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

32. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

33. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

34. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

35. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

36. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

37. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

38. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.

39. The Breast Cancer Genome Atlas Research Network: Ductal carcinoma in situ of the breast: current status. Cancer 2002, 94:1151-1166.
Twist in situ and invasive lobular breast carcinoma. Int J Cancer 2003, 107:970-975.

38. Honorio S, Agathangelou A, Schuermann M, Pankow W, Viacava P, Maher ER, Latif F: Detection of RASSF1A aberrant promoter hypermethylation in sputum from chronic smokers and ductal carcinoma in situ from breast cancer patients. Cancer Res 2003, 63:147-150.

39. Yuan Y, Liu H, Sahin A, Dai JL: Reactivation of SYK expression by inhibition of DNA methylation suppresses breast cancer cell invasiveness. Int J Cancer 2005, 113:654-659.

Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.

39. Yuan Y, Liu H, Sahin A, Dai JL, Douglas DB, Akiyama Y, Carraway H, Belinsky SA, Esteller M, Gabrielson E, Futscher BW, O'Meara MM, Kim CJ, Rennels MA, Lu D, Gruman LM, Seftor RE, Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012, 131:197-205.
Brouwer R, Rijkers E, Siewerts A, Foekens J, van Vroonhoven M, Aerts J, Grosveld F, Lenhard B, Philipsen S. Genome-wide DNA methylation profiling of non-small cell lung carcinomas. 

Environ Mol Biotechnol 2012, 59.

Gu H, Bock C, Mikkelsen TS, Jager N, Smith ZD, Tomazou E, Gnirke A, Lander ES, Meissner A. Genome-scale DNA methylation mapping of clinical samples at single-nucleotide resolution. Nat Methods 2010, 7:133-136.

Kim JH, Dhanasekaran SM, Prensner JR, Cao X, Robinson D, Kalyana-Sundaram S, Huang C, Shankar S, Jing X, Iyer M, Hu M, Sam L, Grasso C, Maher CA, Palanisamy N, Mehra R, Kominosky HD, Siddiqui J, Yu J, Qin ZS, Chinnavay AM. Deep sequencing reveals distinct patterns of DNA methylation in prostate cancer. Genome Res 2011, 21:1028-1041.

Weng Y, Huang TH, Yan PS. Methylated DNA immunoprecipitation and microarray-based analysis: detection of DNA methylation in breast cancer cell lines. Methods Mol Biol 2009, 590:165-176.

Ruike Y, Imanaka Y, Sato F, Shimazaki K, Tsujimoto M. Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. Biomed Genomics 2010, 11:1-17.

Monta S, Takahashi RI, Yamashita R, Toyoda A, Horii T, Kimura M, Fujiyama A, Nakai K, Tajima S, Matoba R, Ochya T, Hatada I. Genome-wide analysis of DNA methylation and expression of microRNAs in breast cancer cells. Int J Mol Sci 2012, 13:8259-8272.

Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CJ. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U.S.A 1992, 89:1827-1831.

Clark SJ, Harrison J, Paul CJ, Frommer M. High sensitivity mapping of methylated cytosines. Nucleic Acids Res 1994, 22:2990-2997.

Cilella S, Shilts L, Baggerly KA, Issa JP, Krahe R. Analysis and quantitative universal Pyrosequencing methylpyrimidine analysis of CpG sites. Biotechniques 2003, 35:146-150.

Tost J, Dunker J, Gut IG. Analysis and quantification of multiple methylation variable positions in CpG islands by Pyrosequencing. Biotechniques 2003, 35:152-156.

Ehrich M, Nelson MR, Stanisens P, Zabeau M, Liloglou T, Xiniananos G, Cantor CR, Field JK, van den Boom D. Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci U.S.A 2005, 102:15785-15790.

Coolen MW, Statham AL, Gardiner-Garden M, Clark SJ. Genomic profiling of CpG methylation and allelic specificity using quantitative high-throughput mass spectrometry: critical evaluation and improvements. Nucleic Acids Res 2007, 35:e119.

Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U.S.A 1996, 93:9821-9826.

Eads CA, Danenberg KD, Kawakami K, Salz LB, Blake C, Shibata D, Danenberg PV, Laird PW. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000, 28:E32.

Kristensen LS, Mikelsa T, Krypuy M, Dobrovic A. Sensitive Melting Analysis after Real-Time- Methylation Specific PCR (SMART-MSP): high-throughput and probe-free quantitative DNA methylation detection. Nucleic Acids Res 2008, 36:e42.