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Promoter hypermethylation in ductal carcinoma *in situ* of the male breast

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Abstract

Ductal carcinoma *in situ* (DCIS) of the male breast is very rare and has hardly been studied molecularly. In males, we compared methylation status of 25 breast cancer-related genes in pure DCIS ($n = 18$) and invasive breast carcinoma (IBC) with adjacent DCIS (DCIS-AIC) ($n = 44$) using methylation-specific multiplex ligation-dependent probe amplification. Results were compared to female breast cancer (BC). There were no significant differences in methylation features between male pure DCIS, DCIS-AIC and IBC after correction for multiple comparisons. In paired analysis of IBC and adjacent DCIS, *CADM1* showed a significantly higher absolute methylation percentage in DCIS ($P = 0.002$). In cluster analysis, two clusters stood out with respectively infrequent and frequent methylation (*GATA5*, *KLLN*, *PAX6*, *PAX5*, *CDH13*, *MSH6* and *WT1* were frequently methylated). Compared to female DCIS, methylation was in general much less common in male DCIS, especially for *VHL*, *ESR1*, *CDKN2A*, *CD44*, *CHFR*, *BRCA2*, *RB1* and *STK11*. In contrast, *THBS1* and *GATA5* were more frequently methylated in male DCIS. In conclusion, there is frequent methylation of *GATA5*, *KLLN*, *PAX6*, *PAX5*, *CDH13*, *MSH6* and *WT1* in male DCIS. Since there was little change in the methylation status for the studied genes from pure male DCIS to DCIS-AIC and IBC, methylation of these seven genes is more likely to occur early in male breast carcinogenesis. Based on the current markers male DCIS seems to be an epigenetically more advanced precursor of male BC, although in comparison to its female counterpart it appears that fewer loci harbor methylation, pointing to differences between male and female breast carcinogenesis with regard to the studied loci.

Key Words

- breast cancer
- DCIS
- male
- methylation
- MS-MLPA

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Introduction

Male breast cancer (BC) is a rare disease accounting for approximately 1% of all breast cancers (Siegel *et al.* 2019). According to the American Cancer Society an estimated 2670 men will develop BC in the United States in 2019, and of these men, approximately 5% will be diagnosed with

pure DCIS making this entity even more rare (Pappo *et al.* 2005, Siegel *et al.* 2019). Because of the low prevalence of this disease, it has been studied to a far lesser extent than its female counterpart. Therapy is still mostly extrapolated from female BC clinical trials, although there is evidence

that male BC differs from female BC on several levels. For example, male BC is diagnosed at older age, men have more advanced disease at the time of diagnosis and there are differences in the distribution of histologic subtypes and molecular characteristics, for instance, regarding gene amplification and epigenetic alterations (Giordano *et al.* 2004, Hill *et al.* 2005, Anderson *et al.* 2010, Kornegoor *et al.* 2012a,b, Lacle *et al.* 2013).

Epigenetic changes, including DNA methylation, play an essential role in carcinogenesis besides established players such as mutations, copy number changes and genetic rearrangements. DNA methylation is a process in which DNA methyltransferase enzymes (DNMTs) add a methyl group to the 5'-carbon of cytosine in the context of cytosine guanine dinucleotides (CpG sites), a process which is associated with gene silencing, thereby causing inactivation of tumor suppressor genes (Plass 2002, Connolly & Stearns 2012). This epigenetic event is of great interest because it is a mark which can be reversed by epigenetic modifiers such as DNMT inhibitors, and therefore, targeting epigenetically silenced tumor suppressor genes can be considered as a therapeutic option in cancer (Connolly & Stearns 2012). In female BC, using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), frequent promoter hypermethylation of *PAX6*, *BRCA2*, *PAX5*, *WT1*, *CDH13* and *MSH* has been described in both DCIS and IBC (Moelans *et al.* 2011). Using the same technique, prognostic value of methylation status conversion of *SFRP4-1* and *HLTF-2* and promoter hypermethylation of *ID4-2*, *SFRP4-1* and *DAPK1* in female BC metastases has been suggested (Schrijver *et al.* 2015). In male BC, promoter hypermethylation was frequent for *MSH6*, *WT1*, *PAX5*, *CDH13*, *GATA5* and *PAX6* and a high overall methylation status of 25 interrogated genes was correlated with poor survival in a group of 108 male BC patients (Kornegoor *et al.* 2012a). Because not every DCIS lesion progresses to IBC and accurately predicting DCIS behavior is still not possible, it is important to identify drivers that control progression to IBC so treatment strategies can be optimized (Sanders *et al.* 2005).

The aim of this study was to investigate male breast carcinogenesis by, for the first time, assessing the methylation status of 25 breast cancer-related genes in male DCIS, and comparing methylation in DCIS adjacent to IBC (DCIS-AIC) in 44 patients to that in male pure DCIS in 18 patients using MS-MLPA. We also correlated methylation in paired and unpaired DCIS and IBC samples and compared our results to previous MS-MLPA data on female DCIS, using a similar kit (Moelans *et al.* 2011).

Materials and methods

Patient material

Unstained slides of formalin fixed paraffin embedded (FFPE) tissue blocks containing DCIS with adjacent IBC ($n=51$) or containing pure DCIS ($n=20$) were collected from a previously selected large male BC cohort (Vermeulen *et al.* 2017, Cardoso *et al.* 2018). This subgroup was selected based on the availability of a tumor tissue block for central pathology review and sufficient tissue for DNA isolation using the unstained slides available to us. Unfortunately, no tissue block or unstained slides containing only normal breast tissue from the same patient was available. Patient and tumor characteristics including age at diagnosis and hormone receptor status (ER α , PgR and HER2) were recorded. Data concerning *BRCA1/2* testing was not available. According to the Dutch legislation, use of anonymous left-over material for scientific purposes is allowed without informed consent if patients have been informed and given the opportunity to opt out, which is part of the standard treatment contract with patients in our hospital. Since we used anonymized archival pathology material which does not interfere with patient care and does not imply the physical involvement of the patient, our study is exempt from ethical approval and informed consent (Wendler 2006).

Hematoxylin and eosin (H&E) slides were reviewed by an experienced pathologist to confirm the diagnosis and to type and grade the IBC according to the World Health Organization and the modified Bloom and Richardson score (Elston & Ellis 1991). ER α , PgR and HER2 were evaluated using immunohistochemistry and scored according to the Allred-score and the ASCO-CAP guidelines (Allred *et al.* 1998, Wolff *et al.* 2013). The areas of interest (pure DCIS, DCIS-AIC and IBC as well as normal tissue in present) were dissected either manually with a sterile scalpel or by laser capture microdissection, using a Zeiss PALM MD3 laser microdissection system, from five consecutive sections (4 μ m) of FFPE tissue blocks. Laser capture microdissection was done in cases with only small areas of DCIS or with abundant inflammatory cells surrounding the area of interest ($n=7$). DNA was extracted by overnight incubation in lysis buffer with proteinase K (10 mg/mL; Roche) at 56°C, followed by boiling for 10 min and centrifugation.

Results from a previous female BC study evaluating methylation status of DCIS and adjacent IBC ($n=33$) using a similar MS-MLPA kit were used to compare 23 genes between female and male DCIS (Moelans *et al.* 2011).

Clinicopathological data of all analyzed male BC cases (IBC, DCIS-AIC and pure DCIS) are shown in Table 1. Hormone receptor status showed perfect concordance (100%) between DCIS and adjacent IBC.

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

MS-MLPA was performed according to the manufacturer's instructions on an ABI 9700 PCR machine (Applied Biosystems) using the ME002-C1 kit (MRC-Holland, Amsterdam, The Netherlands), containing 25 tumor suppressor genes (Supplementary Table 1, see section on supplementary data given at the end of this article). The ME002-C1 kit was chosen based on in-house experience and literature that shows an association between these genes and BC, concerning frequent methylation and prognosis/survival (e.g. *PAX6*; Moelans *et al.* 2011; *BRCA1*; Sharma *et al.* 2009; *ATM*; Ye *et al.* 2007; *TP73*; Marzese *et al.* 2012; *WT1*; Gupta *et al.* 2009, Moelans *et al.* 2011; *GSTP1*; Sharma *et al.* 2009, Cho *et al.* 2012; *CADM1*; Heller *et al.* 2007; *RARB*; Sharma *et al.* 2009, Cho *et al.* 2012, Marzese *et al.* 2012). The principle of MS-MLPA has been described elsewhere (Nygren *et al.* 2005). In summary, MS-MLPA uses probes that are designed for quantifying methylation. These probes contain a restriction site that is recognized by the

methylation-sensitive *HhaI* enzyme. After incubation of two tubes (containing the same DNA sample) with the probe mix, only one of the tubes is incubated with *HhaI*. This tube will only generate a probe amplification product after PCR if the CpG site is methylated. Using the ratio between the signal, incubated with and without *HhaI*, the level of methylation can be calculated. Appropriate negative and positive controls were taken along each MS-MLPA run.

The PCR products were separated by capillary electrophoresis using a 3730 DNA analyzer (Applied Biosystems). Methylation analysis was performed using GeneScan analysis (Applied Biosystems) and Coffalyser Software (MRC-Holland, version 13.1) after the data of each sample was normalized by dividing the signal of each probe by the signal of all reference probes (not degraded by *HhaI*). Then, the ratio of the relative probe peaks of the undigested sample (not containing *HhaI*) and the corresponding digested sample (containing *HhaI*) was calculated for each probe to obtain the final methylation status. A promoter methylation ratio above 0.15 (corresponding to >15% methylation) was considered as promoter hypermethylation, based on previous studies (Moelans *et al.* 2011, Kornegoor *et al.* 2012a). The mean methylation index (MMI) was defined as the number of methylated genes divided by the number of genes tested.

Table 1 Baseline characteristics of investigated male invasive breast carcinomas, DCIS adjacent to invasive carcinoma and pure ductal carcinoma *in situ*.

	Invasive breast carcinoma <i>n</i> = 44	Adjacent DCIS <i>n</i> = 44	Pure DCIS <i>n</i> = 18
Age (years)			
Mean (range)	63.3 (37–85)	63.2 (37–85)	62.6 (37–76)
Histologic subtype IC			
Ductal type carcinoma	41 (93.2%)		
Mucinous carcinoma	1 (2.3%)		
Micropapillary carcinoma	1 (2.3%)		
Mixed type			
Ductal/micropapillary	1 (2.3%)		
Grade			
1	14 (31.8%)	11 (25.0%)	3 (16.7%)
2	19 (43.2%)	27 (61.4%)	14 (77.8%)
3	11 (25.0%)	6 (13.6%)	1 (5.6%)
ER			
Positive	44 (100%)	44 (100%)	18 (100%)
Negative	0 (0%)	0 (0%)	0 (0%)
PR			
Positive	42 (95.5%)	42 (95.5%)	18 (100%)
Negative	2 (4.5%)	2 (4.5%)	0 (0%)
HER2			
Positive	1 (2.3%)	1 (2.3%)	1 (5.6%)
Negative	43 (97.7%)	43 (97.7%)	17 (94.4%)

Statistics

Statistical calculations were done using SPSS for Windows, version 21.0. MMI in pure DCIS and DCIS-AIC was compared using the Mann–Whitney test (unpaired data) and in DCIS-AIC and IBC using the Wilcoxon signed-ranks test (paired data). Individual gene comparison was done using the Mann–Whitney test (absolute methylation percentages) and chi-square test (dichotomized data) in pure DCIS and DCIS-AIC and using the Wilcoxon signed-ranks test (absolute methylation percentages) and McNemar test (dichotomized data) in paired DCIS-AIC and IBC.

The differences between female and male DCIS and differences in histologic grades in DCIS-AIC and IBC were compared using Mann–Whitney and chi-square tests for absolute methylation percentages and dichotomized data, respectively. *P* values below 0.05 were considered significant and correction for multiple comparisons was done using the Holm–Bonferroni method. Finally, unsupervised hierarchical clustering (Euclidian distance method) of absolute methylation percentages was performed using the statistical program R (www.r-project.org) for male cases (female cases previously described; [Moelans *et al.* 2011](#)).

Results

Methylation status of male DCIS and IBC

Eighteen cases of pure DCIS and 44 cases of DCIS-AIC and matched IBC had sufficient DNA yield for further analysis. Using absolute methylation percentages, *CADM1* showed a significantly higher methylation percentage in DCIS-AIC (mean 12.3%) compared to IBC (mean 9.0%) (paired analysis, *P*=0.002) and significance remained after correction for multiple comparisons. Absolute methylation percentages of *PAX5* and *CDH13* were significantly higher in DCIS-AIC (mean 28.1 and 29.0%, respectively) compared to pure DCIS (20.0 and 18.0%, respectively) (*P*=0.018 for both). These findings however, lost their significance after correction for multiple comparisons.

Using the 15% threshold, no significant differences were found for individual genes between pure DCIS vs DCIS-AIC and DCIS-AIC vs IBC in dichotomized unpaired analysis. In paired analysis of IBC and DCIS-AIC (*n*=44) using this 15% threshold, *MGMT* showed a significantly higher percentage of methylated cases in IBC (25% of cases) compared to DCIS-AIC (9.1% of cases) (*P*=0.039), but significance was lost after correction for multiple comparisons. In this analysis, *CADM1* showed

no significant difference in number of cases with >15% methylation between DCIS-AIC and IBC (*P*=0.219).

Table 2 shows methylation frequencies for all 25 interrogated genes. **Figure 1** illustrates the methylation frequencies in male DCIS and IBC. There was no significant difference in the number of methylated genes between pure DCIS and DCIS-AIC (*P*=0.402) and between DCIS-AIC and IBC (*P*=0.205), all having an average of seven methylated genes. The number of methylated genes varied between 2 and 10 for pure DCIS, 1 and 11 for DCIS-AIC and 3 and 13 for IBC. The MMI was 0.26 for pure DCIS, 0.28 for DCIS-AIC and 0.30 for IBC (*P*=0.240 and *P*=0.205 for pure DCIS vs DCIS-AIC and DCIS-AIC vs IBC, respectively).

Correlation of methylation with tumor grade

The percentage of methylated cases and absolute methylation percentages were compared between low/intermediate-grade and high-grade DCIS-AIC and between low/intermediate-grade and high-grade IBC. *PAX6* and *CDH13* showed higher absolute methylation percentages

Table 2 Comparison of percentage of methylated cases (cut-off value 15%) of the 25 analyzed genes in pure ductal carcinoma *in situ* (DCIS), DCIS with adjacent invasive carcinoma (DCIS-AIC) and the adjacent invasive breast carcinoma (IBC) in males.

Gene	Hypermethylation (%)			Chromosome
	Pure DCIS	DCIS-AIC	IBC	
<i>MSH6</i>	100	98	100	2p16.3
<i>KLLN</i>	89	91	89	10q23.31
<i>GATA5</i>	83	75	89	20q13.33
<i>WT1</i>	78	75	82	11p13
<i>PAX5</i>	44	68	75	9p13.2
<i>THBS1</i>	72	57	64	15q14
<i>CDH13</i>	39	70	64	16q23.3
<i>PAX6</i>	22	36	43	11p13
<i>GSTP1</i>	11	32	41	11q13.2
<i>MGMT</i>	22	9	25	10q26.3
<i>TP53</i>	22	27	18	17p13.1
<i>TP73</i>	11	16	14	1p36.32
<i>ESR1</i>	6	5	9	6q25.1
<i>CD44</i>	0	7	9	11p13
<i>CADM1</i>	11	16	7	11q23.3
<i>RARB</i>	11	2	5	3p24.2
<i>BRCA2</i>	11	5	2	13q13.1
<i>PYCARD</i>	6	2	2	16p11.2
<i>VHL</i>	0	0	0	3p25.3
<i>CDKN2A</i>	6	0	0	9p21.3
<i>ATM</i>	0	0	0	11q22.3
<i>CHFR</i>	0	0	0	12q24.33
<i>RB1</i>	0	0	0	13q14.2
<i>BRCA1</i>	0	0	0	17q21.31
<i>STK11</i>	6	0	0	19p13.3

Figure 1
Percentage of cases showing >15% methylation concerning 25 breast cancer-related genes in pure ductal carcinoma *in situ* (DCIS), DCIS with adjacent invasive carcinoma (DCIS-AIC) and the adjacent invasive breast carcinoma (IBC) in males.

in high grade DCIS-AIC, but there was no significance after correction for multiple comparisons ($P=0.025$ and $P=0.033$, respectively). No significant differences in percentage of methylated cases (binary) were found for these genes between low/intermediate-grade and high-grade DCIS-AIC.

In IBC the genes *PAX5*, *PAX6*, *GSTP1*, *ATM* and *GATA5* showed a similar pattern but were also not significantly different after correction for multiple comparisons ($P=0.004$, $P=0.005$, $P=0.047$, $P=0.036$, $P=0.038$, respectively). Of these genes, only *PAX6* showed a significant difference in percentage of methylated cases between low/intermediate-grade and high-grade IBC ($P=0.04$), which was also lost after correction for multiple comparisons.

The average number of methylated genes was not significantly different between grades for DCIS-AIC or IBC after correction for multiple comparisons.

Cluster analysis

Figure 2 shows the results of unsupervised hierarchical cluster analysis of 18 interrogated genes in all pure DCIS, DCIS-AIC and IBC cases. The 18 genes that showed the highest spread in absolute methylation percentage were included in the cluster analysis. This was done to prevent that less variable genes may blur and influence the results. There were three recognizable gene clusters. One cluster (cluster A) consisted of *GATA5*, *KLLN*, *PAX5*, *CDH13*, *MSH6*, *PAX6* and *WT1*, all frequently methylated (>43% hypermethylated cases in IBC). A second cluster (cluster B) was formed by genes that were less frequently methylated, varying between 2 and 25% methylated cases in IBC and consisted of *MGMT*, *TP73*, *TP53*, *BRCA2*, *ESR1*, *CADM1*, *THBS1*, *CD44* and *RARB*. A small cluster (cluster C) only consisting of *GSTP1* and *PYCARD* showed variable levels of methylation, with averages of 41 and

2% hypermethylated IBC cases, respectively. For male BC cases two large clusters (cluster I and cluster II) could be identified with 20 IBC cases in cluster I and 24 IBC cases in cluster II. Thirty-two DCIS-AIC with corresponding IBC cases clustered together in cluster I and cluster II. Age, mitoses (MAI) and grade were not significantly different between IBC in these two clusters.

Comparison of methylation status between male and female DCIS

Methylation was much less common in male DCIS-AIC for a variety of genes. After correction for multiple comparisons, the genes *VHL*, *ESR1*, *CDKN2A*, *CD44*, *CHFR*, *BRCA2*, *RB1* and *STK11* showed a significantly lower absolute methylation percentage in male DCIS-AIC (all P values equal or smaller than 0.002). *THBS1*, *MSH6* and *GATA5* showed significantly higher absolute methylation percentages ($P<0.001$, $P<0.001$ and $P=0.001$, respectively).

Of the genes showing significance in absolute methylation percentage analysis, *ESR1*, *BRCA2* and *STK11* also showed a significant lower percentage of methylated cases in male DCIS-AIC compared to female DCIS-AIC ($P=0.002$, $P<0.001$ and $P<0.001$, respectively) and *THBS1* and *GATA5* a higher percentage of methylated cases in male DCIS-AIC compared to female DCIS-AIC ($P=0.002$ and $P=0.002$, respectively). Figure 3 illustrates the percentage of methylated cases of the same 23 interrogated genes (identical CpG sites and technique) in male and female DCIS-AIC.

Discussion

To investigate the role of promoter hypermethylation in male BC during tumor progression, we compared the methylation status of 25 breast cancer-related tumor

Figure 2

Hierarchical cluster analysis showing five gene clusters and two clusters of cases in pure ductal carcinoma *in situ*, ductal carcinoma *in situ* adjacent to invasive carcinoma and invasive carcinoma of the male breast and two clusters of BC cases.

suppressor genes between 18 cases of pure DCIS and 44 cases of DCIS-AIC and between these DCIS-AIC cases and paired IBC using MS-MLPA. In addition to the absolute methylation percentages, we used dichotomized data with a cut-off of 15% methylation as established in previous studies.

For methylation detection, multiple methods are available such as methylation-specific PCR (MSP) or quantitative multiplex MSP (QM-MSP). We chose MS-MLPA as this is a robust, inexpensive and easy-to-perform technique. In comparison to MSP, it uses

methylation-sensitive HhaI enzyme instead of sodium bisulfite conversion of unmethylated cytosine residues, which is known to be a difficult part in the process to standardize and can lead to DNA degradation (Jeuken *et al.* 2007, Moelans *et al.* 2018). In addition, MS-MLPA has shown good correlation with QM-MSP in a study comparing different techniques in BC (Suijkerbuijk *et al.* 2010, Furlan *et al.* 2013, Lopez *et al.* 2014).

Most IBCs were ductal carcinoma NOS and most tumors were grade 2, consistent with literature

Figure 3

Percentage of cases showing >15% methylation concerning 22 breast cancer-related genes in male and female ductal carcinoma *in situ* (DCIS). Genes with an asterisk were significantly different for both the absolute methylation percentage and percentage of methylated cases, between male and female DCIS after correction for multiple comparisons. P-values for methylation status are shown.

(Kornegoor *et al.* 2012a, Vermeulen *et al.* 2017). Using absolute methylation percentages, promoter methylation in pure DCIS vs DCIS-AIC and DCIS-IBC vs IBC was overall not significantly different after correction for multiple comparisons. Using the 15% threshold, there was no significant difference between pure DCIS vs DCIS-AIC and DCIS-AIC vs IBC in the number of methylated tumor suppressor genes. Previous female BC studies showed similar methylation levels in DCIS and IBC, in line with our findings (Moelans *et al.* 2011, Park *et al.* 2011, Verschuur-Maes *et al.* 2012). In the current study *CADM1* had a significantly higher absolute methylation percentage in DCIS-AIC compared to the adjacent IBC, although the percentage of methylated cases using the 15% threshold was not significantly different. *CADM1* (cell adhesion molecule 1) is a tumor suppressor gene located at chromosome 11q23.2 and encodes a membrane glycoprotein that plays a role in cell adhesion. It has been described to be frequently inactivated in various tumors such as non-small-cell lung carcinoma (Fukami *et al.* 2003). In BC, *CADM1* promoter methylation was found in 23/50 (46%) primary BCs using pyrosequencing (Takahashi *et al.* 2012). Lower methylation levels in more advanced lesions, such as IBC compared to DCIS, may be explained by the reversibility of hypermethylation and tumor heterogeneity and has previously been described in female BC, where *CADM1* showed a significantly lower methylation frequency using a 15% cut-off in BC metastases compared to the primary tumor (Schrijver *et al.* 2015).

In at least 60% of both DCIS-AIC and IBC lesions methylation was high for *KLLN*, *WT1*, *CDH13*, *MSH6*, *GATA5* and *PAX5*, genes that also clustered together in our cluster analysis, together with *PAX6*. Of these genes, *WT1*, *CDH13*, *MSH6*, *GATA5* and *PAX5* were shown to be frequently methylated (>50%) in a previous male BC study and *WT1*, *CDH13*, *MSH6* and *PAX5* in a previous female BC study (Moelans *et al.* 2011, Kornegoor *et al.* 2012a). *KLLN* was not included in the MS-MLPA kit used in these previous studies. This indicates that methylation of these genes, especially *WT1*, *CDH13*, *MSH6*, *GATA5* and *PAX5*, could play an important role in BC, and specifically in male breast carcinogenesis. Methylation of these genes seems to be an early event in breast carcinogenesis as we saw no significant differences between DCIS and IBC.

A limitation of this study is that we were unable to analyze the normal breast tissue in the samples. Only six samples had enough surrounding normal tissue left, which was mostly fat and in close proximity to the abnormal tissue. DNA concentration of these samples was

not sufficient and MS-MLPA results were therefore not reliable. MS-MLPA analysis on normal male breast tissue was done in the previous male BC study by Kornegoor *et al.*, where normal male breast tissue ($n=10$) derived from autopsies showed low methylation levels in 25 genes. Only *MSH6*, *ESR1*, *PAX5* and *CDH13* showed methylation (40, 20, 10 and 10%, respectively) (Kornegoor *et al.* 2012a). In addition, a previous male BC study investigating promoter methylation of six genes including *ESR1* using pyrosequencing found higher methylation levels in male IBC ($n=69$) compared to the median methylation levels in normal breast samples ($n=7$) (Rizzolo *et al.* 2018). This finding supports the assumption that methylation of several genes is an early event and that these genes are not likely to be silenced in normal male BC tissue. Genes that were rarely methylated in our study were *PYCARD*, *CHFR* and *CDKN2A*, of which the latter two also showed low methylation (<2%) in the male BC study by Kornegoor *et al.* (Kornegoor *et al.* 2012a). We did not compare protein expression by immunohistochemistry to MS-MLPA results since previous studies have shown only weak concordance between immunohistochemistry and methylation (Jeuken *et al.* 2007, Furlan *et al.* 2013, Lopez *et al.* 2014, Mao *et al.* 2016).

In this study, we observed several differences between low/intermediate- and high-grade IBC and between low/intermediate- and high-grade DCIS-AIC. In high-grade IBC, genes *PAX5*, *PAX6*, *GSTP1*, *ATM* and *GATA5* showed a higher absolute methylation frequency and a higher percentage of cases showing *PAX6* methylation was seen in high-grade IBC. In DCIS-AIC, *PAX6* and *CDH13* showed a higher absolute methylation percentage in high-grade lesions. These findings could indicate that methylation of these genes may be important in the development of these high-grade lesions.

Compared to females, methylation levels for *VHL*, *ESR1*, *CDKN2A*, *CD44*, *CHFR*, *BRCA2*, *RB1* and *STK11* were significantly lower in male DCIS-AIC after correction for multiple comparisons. In addition, *ESR1*, *BRCA2* and *STK11* also showed a significantly lower methylation frequency in male versus female DCIS-AIC.

Estrogen receptor α (ER α) is a transcription factor encoded by *ESR1*. Activation of ER α plays a role in cell proliferation and differentiation in various cells (Lin *et al.* 2007). In BC, ER α negativity is associated with a poor response to endocrine therapy and a poor prognosis (Duffy *et al.* 2017).

In female BC, similar methylation levels have been described between DCIS and IBC (Moelans *et al.* 2011). Our finding of lower methylation levels of *ESR1* in male

DCIS compared to female DCIS is in line with previous findings in male IBC (Kornegoor *et al.* 2012a). This finding points to possible differences in male and female breast carcinogenesis concerning promoter methylation.

BRCA2 encodes for the protein BRCA2 and is involved in DNA repair (Xia *et al.* 2006). The lower *BRCA2* methylation levels in male versus female DCIS may be explained by the higher rate of *BRCA2* mutation carriers in the male BC population. *BRCA2* mutations occur in 4–40% of male BC and in approximately 3.7% of female BC (King *et al.* 2003, Ottini *et al.* 2010). *BRCA1/2* methylation as a ‘second hit’ has been described to be an infrequent event in *BRCA1/2* mutation carriers, although a recent systematic review by Vos *et al.* could not support this assumption (Dworkin *et al.* 2009, Vos *et al.* 2017, 2018). Unfortunately, no data regarding *BRCA2* mutation status were available. Germline mutations in *STK11*, encoding LKB1, are associated with Peutz-Jeghers syndrome, a syndrome associated with a 24–54% lifetime risk of developing BC (Beggs *et al.* 2010). In sporadic BC *STK11* mutations are rare but hypermethylation of this tumor suppressor gene has been described in papillary breast carcinomas (Esteller *et al.* 2000).

THBS1, *GATA5* and *MSH6* showed higher methylation levels in male DCIS-AIC compared to females, of which the former two (*THBS1* and *GATA5*) also showed a higher methylation status in men. *THBS1* encodes TSP-1 (thrombospondin 1), a glycoprotein that plays a role in angiogenesis inhibition, leading to suppression of tumor growth (Ren *et al.* 2006). In the male BC study by Kornegoor *et al.*, no significant differences were found in *THBS1* methylation between male and female BC, so these results should be interpreted with caution and should be confirmed in a larger male BC cohort (Kornegoor *et al.* 2012a).

GATA factors are a family of transcription regulatory proteins and *GATA4*, *GATA5* and *GATA6* regulate the development of endoderm-derived organs. Loss of *GATA5* expression due to promoter hypermethylation has been reported in a variety of cancers, such as colorectal, gastric, lung and ovarian cancer (Akiyama *et al.* 2003, Guo *et al.* 2004, Hellebrekers *et al.* 2009, Peters *et al.* 2014). In colorectal cancer *GATA5* has been described as an important tumor suppressor gene that is hypermethylated early during carcinogenesis (Hellebrekers *et al.* 2009).

In conclusion, there is relatively frequently methylation of *GATA5*, *KLLN*, *PAX5*, *CDH13*, *MSH6* and *WT1* in male DCIS. Since there was little difference in methylation status for common tumor suppressor genes from pure male DCIS to DCIS-AIC and IBC, methylation of

these six genes could play an important early role in male breast carcinogenesis. Male DCIS seems to be a genetically advanced precursor of male BC, although it seems to harbor less methylation than its female counterpart, pointing to differences in male and female breast carcinogenesis.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0485>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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