original article

Methylation is less abundant in BRCA1-associated compared with sporadic breast cancer

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Background: Promoter methylation is a common epigenetic mechanism to silence tumor suppressor genes during breast cancer development. We investigated whether BRCA1-associated breast tumors show cancer-predictive methylation patterns similar to those found in sporadic tumors.

Patients and methods: Quantitative multiplex methylation-specific PCR of 11 genes involved in breast carcinogenesis (*RARB, RASSF1, TWIST1, CCND2, ESR1, SCGB3A1, BRCA1, BRCA2, CDKN2A, APC, CDH1*) was carried out on 32 BRCA1-associated and 46 sporadic breast carcinomas and on normal breast tissue from seven BRCA1 mutation carriers and 13 non-carriers.

Results: The extent of cumulative methylation increased with age ($P < 0.001$). The median cumulative methylation index (CMI) of all studied genes was significantly higher in tumors (89) than in normal tissue (13, $P < 0.001$). The median CMI was significantly lower in BRCA1-associated (59) than in sporadic breast tumors (122, $P = 0.001$), in estrogen receptor (ER)-negative tumors (73) than in ER-positive tumors (122, $P = 0.005$) and in lymph node-negative (77) compared with lymph node-positive tumors (137, $P = 0.007$). In subgroup analysis, the effect of a BRCA1 germline mutation on methylation proved to be independent of ER status, lymph node status and age.

Conclusions: These data indicate that BRCA1-associated breast cancers show less promoter methylation compared with sporadic breast carcinomas indicating a difference in disease etiology.

Key words: BRCA1, breast cancer, hereditary, methylation, QM-MSP

introduction

With 1 million new cases causing 375 000 deaths worldwide per year, breast cancer is the leading cause of cancer death in women in both developing and developed countries [1]. Several hormonal and lifestyle factors have been identified as risk factors for breast cancer, but the best established risk factor is the presence of germline-mutated BRCA1 or BRCA2 genes, which indicates a lifetime risk of 45%–85% to develop breast cancer [2, 3].

Regular screening by clinical breast examination, mammography and magnetic resonance imaging (MRI) is offered to these women, but has not been shown to significantly reduce breast cancer mortality [4]. Although the discriminating capacity of MRI is higher than that of mammography, considerable false-positive rates are encountered, increasing with higher breast density and younger age [5]. Hence, there is an urgent need for molecular biomarkers that detect hereditary breast cancer at an early, preferably even pre-invasive, stage.

Methylation patterns may serve as biomarkers that might very well be suitable to achieve this. DNA methylation is a cell heritable epigenetic modification that plays essential roles during (post-implantation) developmental processes such as X-chromosome inactivation, genomic imprinting [6] and also tumor development [7, 8]. Cancer cells are characterized by methylation imbalance where genome-wide hypomethylation [9] is accompanied by localized hypermethylation of CpG islands and increases in the expression of DNA methyltransferases [10]. DNA hypermethylation is associated with aberrant silencing of transcription, potentially causing a first or second hit and in this way contributing to tumor suppressor gene inactivation [10, 11]. There is mounting evidence that breast cancer develops by gradual accumulation of interacting epigenetic and genetic events [10, 12]. Whereas the mutation spectra may differ greatly between patients, epigenetic alterations like methylation are thought to occur in a more global manner during carcinogenesis [7, 8]. Moreover, methylation plays an early role in tumor development and is therefore a promising biomarker for the early detection of malignancy [13, 14].

The arduous search for biomarkers with diagnostic and prognostic value indicates that the predictive value of a single

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marker might not be sufficiently discriminatory. This likely also applies to methylation, so an approach assessing multiple genes involved in different biological processes (e.g. DNA repair, cell cycle control, adherence and apoptosis) is to be preferred. Quantitative multiplex methylation-specific PCR (QM-MSP) seems particularly suitable for this application, since this method permits simultaneous quantitative assessment of methylation in multiple genes using very small quantities of DNA. Indeed, the use of QM-MSP for analyzing promoter hypermethylation in ductal fluids has been proven to be successful in the detection of sporadic (i.e. due to a non-BRCA-associated carcinogenetic pathway) breast cancer [15] and in distinguishing between tumor and normal tissues using paraffin-embedded archival tissues [16]. On the basis of prior findings in the intestine, where hereditary defects in mismatch repair induced promoter hypermethylation [17] indicating a link between methylation and genetic instability due to defects in DNA repair [18], we hypothesized that the extent of methylation might be different in BRCA1-related breast cancer compared with sporadic cases. In order to evaluate the potential use of methylation as a biomarker in screening women with a hereditary predisposition to breast cancer, we applied QM-MSP to compare promoter hypermethylation in BRCA1-associated breast tumors to sporadic tumors, using a validated panel of target genes for which methylation has been proven to be an accurate marker of sporadic breast malignancy [i.e. RARB, RASSF1, TWIST1, CCND2, SCGB3A1 (also known as HIN1), BRCA1, BRCA2, CDKN2A, APC] [15, 16]. CDH1 (E-cadherin) and estrogen receptor (ER) alpha (ESR1) were added to the panel because they have been reported to be epigenetically inactivated in breast cancer [19, 20].

methods

patients and pathology

Thirty-two invasive breast carcinomas from BRCA1 germline mutation carriers and 46 sporadic breast tumors were obtained from the Pathology Department of the University Medical Center, Utrecht. Thirteen reduction mammoplasty specimens (sporadic control) and seven prophylactic mastectomy specimens (BRCA1 germline mutation carrier control) containing only histologically normal breast epithelium were used as controls. Specimens from BRCA1 mutation carriers (collected between 1983 and 2005) were selected on the basis of availability and sporadic samples were randomly selected. Use of anonymous or coded leftover material for scientific purposes is part of the standard treatment contract with patients in our hospital [21].

Hematoxylin–eosin-stained slides of the paraffin blocks were reviewed by a single pathologist (PvD) to confirm the presence of malignancy in tumor samples. The percentage of carcinoma cells in each tissue section was estimated to be at least 50%. Histologic type was assessed according to the World Health Organization. Mitotic index was determined as described [22], grade was assessed according to the Nottingham system [23] and the ER, progesterone receptor (PR) and HER-2/neu receptor were assessed by standard immunohistochemistry [24].

DNA isolation

A 10-μm unstained section was deparaffinized by treatment of 2× 5-min xylene, and the relevant tissue was scraped from the slide. Fifty microliters of TNES (10 mM Tris/150 mM NaCl/2 mM EDTA/0.5% sodium dodecyl sulfate) extraction buffer containing 250 ng salmon sperm DNA (Invitrogen Corp., Carlsbad, CA) and 100 μg proteinase K (Invitrogen Corp.) was added to the tissue. After 4-h rotation at 52°C, samples were heat inactivated for 5 min at 99°C and stored at 4°C.

Quantitative multiplex methylation-specific PCR

QM-MSP was carried out as described by Fackler et al. [15, 16]. Briefly, 13.5 μl of isolated DNA was heated at 99°C for 10 min, quick chilled on ice after which 1.5 μl of 2 M NaOH (freshly prepared) was added. Thirty-five microliters of 4.5 M sodium bisulfite (Sigma-Aldrich, St. Louis, MO, USA) containing 1 mM hydroquinone (Sigma; both freshly prepared, mixed just before adding) was added to the sample, after which it was kept 4 h at 55°C in the dark under oil.

Microspin ion-exchange columns (Amersham Biosciences, Piscataway NJ, USA) were used for purification according to the manufacturer's directions. After a 5-min incubation, a mixture of 212 μl of H_2O, 130 μl of 10 M NH_4OAc, 3 μl of glycogen and 1 ml of absolute ethanol was used for precipitation (at −20°C overnight). The next day samples were centrifuged 30 min 16000 g at 4°C, drained and washed with 75% ethanol, after which the pellet was dissolved in 5 μl H_2O.

The QM-MSP procedure requires two sequential PCR reactions [16]. Five microliter dissolved DNA was multiplexed in a 50-μl PCR reaction using MSP buffer (16.6 mM NH_4SO_4, 67 mM Tris, pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% dimethyl sulfoxide), 0.0625 mM deoxyribonucleotide triphosphates (dNTP), 0.1 μM $MgCl_2$, 10 U Platinum Taq and 100 ng of each reverse and forward primer. The external (non-CpG-dependent) primers for RARB, RASSF1, TWIST1, CCND2, SCGB3A1, BRCA1, BRCA2, CDKN2A and APC were as previously described [15, 16]. For ESR1 and CDH1, primer sequences are in the supplementary material section (available online). The PCR conditions were as follows: 95°C for 5 min, 36 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 45 s, followed by an extension cycle of 72°C for 5 min. Human sperm DNA (HSD) was used as a negative control, SssI treated MDA-MB-231 cells or treated HSD as a positive control. The PCR products were diluted 1:5 with water and stored at −20°C.

Two microliters of 1:5 diluted multiplexed DNA were used for real-time PCR in a final volume of 25 μl, using 2.5 μl 10× MSP buffer (as above), 200 μM dNTP, 1.25 U Ramp Taq (Denville Scientific, Metuchen, NJ, USA) and 1× ROX (Invitrogen). A gene-specific primer and probe set [15, 16] containing 600 nM each of two primers (forward and reverse) and 200 nM labeled probe (Applied Biosystems, Foster City, CA, USA) was added to the reaction mix. The internal primers and probes for RARB, RASSF1, TWIST1, CCND2, SCGB3A1, BRCA1, BRCA2, CDKN2A and APC were as previously described [15, 16], except for CDKN2A RT-FM(3) cggatcgcgtgcgttcggcg. Primers and probes for ESR1 and CDH1 are in the supplementary material section (available online). PCR conditions were 95°C for 7 min, followed by 40 cycles of 95°C for 15 s and 65°C for 1 min. A standard curve (dilutions $10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}$) and a 80-K copy number control were included for extrapolating percent methylation from the U and M curves similar to the 40-K control described previously [16].

statistical analysis

The cumulative methylation index (CMI) was calculated as the sum of the percentage methylation for all genes. For example, for 11 genes 100% methylation × 11 genes = 1100.

Mann–Whitney and Kruskal–Wallis tests were used for comparing medians between groups. The chi-square test was used for comparing proportions. Spearman correlation coefficients were computed for assessing the association between methylation and other continuous variables. Logistic regression was used to adjust the association between methylation (as a continuous variable) and malignancy or the presence of a BRCA1 germline mutation for age as a confounding factor.

SPSS 12.0.1 for Windows was used for statistical analysis. Statistical tests were considered statistically significant at two-sided $P \leq 0.05$.

results

baseline characteristics of study cohort

Median age at the time of breast surgery was 45 years. Median age was significantly lower for patients undergoing reduction mammoplasty (26 years) or prophylactic mastectomy (40 years) versus tumor-associated operations ($P < 0.001$) and for BRCA1-associated tumors (39 years) versus sporadic (58 years) tumors ($P = 0.001$).

Most BRCA1-associated tumors were of the ductal type (26 of 32); the other BRCA1-associated tumors showed lobular (1 of 32), medullary (4 of 32) or metaplastic histology (1 of 32). The sporadic cancers were also mostly ductal carcinomas (37 of 46), but medullary cancers were slightly less (1 of 46) and tubular (1 of 46), lobular (3 of 46) and mucinous (4 of 46) tumors slightly more frequent than among the BRCA1-associated tumors.

BRCA1-associated tumors were significantly more often ER ($P < 0.001$), PR ($P = 0.001$) and HER2/neu negative ($P < 0.01$) than sporadic tumors and had a higher grade ($P < 0.001$) and a higher mitotic index ($P < 0.001$). Lymph node status did not vary significantly between BRCA1-associated and sporadic tumors. All baseline characteristics are shown in Table 1.

methylation in BRCA1-associated compared with sporadic tumors

The extent of methylation of the various genes is specified in Table 2. The median CMI was significantly higher in tumors (89) than in normal tissue (13, $P < 0.001$). The median CMI was significantly lower in BRCA1-associated (59) than sporadic breast tumors (122, $P = 0.001$). The median CMI in normal breast tissue was higher for BRCA1 mutation carriers (31) compared with non-carriers (7, $P = 0.03$).

methylation in ER-negative compared with ER-positive tumors and in lymph node-positive compared with lymph node-negative tumors

The median CMI was significantly lower in ER-negative tumors (73) than ER-positive tumors (122, $P = 0.005$). When examining BRCA1-associated and sporadic tumors separately, there was a significant difference in median CMI between the BRCA1-associated ER-negative (46) and ER-positive tumors (118, $P = 0.03$), while the median CMI for the sporadic ER-negative and ER-positive tumors (117 and 126, respectively) were not significantly different. ESR1 methylation was not higher in ER-negative compared with ER-positive tumors; this did not change with stratification for the presence of a BRCA1 germline mutation.

The median CMI was significantly higher in lymph node-positive (137) than in lymph node-negative tumors (77, $P = 0.007$).

correlation with age

The extent of methylation was positively correlated to age (Spearman correlation coefficient 0.46; $P < 0.001$). The

Table 1. Baseline characteristics for sporadic and BRCA1-asscociated breast tumors

	Sporadic tumor Median (IQR[a])	BRCA1-associated tumor Median (IQR[a])
N	46	32
Age (years)[b]	58 (47–73)	39 (34–48)[c]
Mitotic index (mitoses/2 mm²)[d]	13 (4–33)	33 (20–48)[c]

	Sporadic tumor % (n)	BRCA1-associated tumor % (n)
Histology		
Ductal	80 (37)	81 (26)
Lobular	7 (3)	3 (1)
Medullary	2 (1)	13 (4)
Mucinous	9 (4)	0 (0)
Metaplastic	0 (0)	3 (1)
Tubular	2 (1)	0 (0)
Estrogen receptor[d]		
Negative	28 (13)	91 (29)
Positive	72 (33)	9 (3)[e]
Progesterone receptor[d]		
Negative	44 (19)	84 (27)
Positive	57 (26)	16 (5)[e]
HER-2/neu[d]		
Negative	82 (37)	100 (31)
Positive	18 (8)	0 (0)[f]
Grade[d]		
1	24 (11)	0 (0)
2	33 (15)	13 (4)
3	44 (20)	88 (28)[e]
Lymph node status[d]		
Negative	44 (20)	57 (16)
Positive	57 (26)	43 (12)

[a]IQR , interquartile range: 25th–75th percentile.
[b]Median and numbers for normal tissue samples (reduction mammoplasty and prophylactic mastectomy) are given in the 'Results' section.
[c]Difference of medians is statistically significant ($P \leq 0.001$; Mann–Whitney).
[d]Data not available for all subjects.
[e]Difference in distribution is statistically significant ($P \leq 0.001$; chi square).
[f]Difference in distribution is statistically significant ($P \leq 0.01$; chi square).

association between methylation (CMI) and the presence of malignancy remained when adjusted for age ($P = 0.04$). In a multivariate logistic regression model, age and the presence of a BRCA1 germline mutation ($P < 0.05$) were independent determinants of CMI. CMI was not associated with tumor grade, histologic type, PR or HER-2/neu status and was not correlated to mitotic index.

subgroup analysis

When the analysis was restricted to the lymph node negative, ER negative or women under the age of 45 years, the differences in CMI between the sporadic and BRCA1-associated tumors

Table 2. Methylation (medians and means) for different genes by Quantitative Multiplex Methylation-Specific PCR according to BRCA1 germline mutation status and tumor presence

Gene	Sporadic Normal[a] n = 13 Median (IQR[c])	Mean (SE)	Tumor n = 46 Median (IQR[c])	Mean (SE)	BRCA1 associated Normal[b] n = 7 Median (IQR[c])	Mean (SE)	Tumor n = 32 Median (IQR[c])	Mean (SE)	All Total normal n = 20 Median (IQR[c])	Mean (SE)	Total tumor n = 78 Median (IQR[c])	Mean (SE)
RARB	2% (0–5)	7% (4)	7% (1–22)	15% (3)	4% (1–6)	4% (1)	5% (2–13)	10% (3)	2% (0–6)	6% (3)	6% (2–17)[d]	13% (2)
RASSF1	0% (0–3)	2% (1)	18% (1–44)[e]	26% (4)	1% (0–1)	1% (0)	0% (0–4)[f]	9% (4)	1% (0–2)	2% (1)	4% (0–36)	19% (3)
TWIST1	1% (0–1)	1% (1)	14% (2–63)[e]	30% (5)	1% (0–5)	2% (1)	2% (0–19)[g]	17% (5)	1% (0–1)	1% (1)	6% (1–49)[e]	25% (4)
CCND2	0% (0–0)	0% (0)	1% (0–30)[e]	14% (3)	0% (0–1)	0% (0)	0% (0–4)	11% (5)	0% (0–0)	0% (0)	0% (0–16)[e]	13% (3)
ESR1	0% (0–0)	2% (1)	0% (0–0)	1% (0)	0% (0–3)	4% (3)	0% (0–1)	5% (3)	0% (0–1)	2% (1)	0% (0–0)	2% (1)
SCGB3A1	0% (0–0)	0% (0)	19% (1–67)[e]	35% (6)	13% (0–32)[g]	26% (15)	0% (0–0)[d,f]	6% (4)	0% (0–1)	8% (5)	0% (0–40)	23% (4)
BRCA1	0% (0–4)	5% (3)	0% (0–1)	5% (2)	0% (0–5)	9% (8)	0% (0–22)	14% (4)	0% (0–6)	7% (3)	0% (0–2)	8% (2)
BRCA2	0% (0–0)	1% (0)	0% (0–0)	2% (1)	0% (0–4)	12% (10)	0% (0–0)	3% (2)	0% (0–2)	5% (4)	0% (0–0)	2% (1)
CDKN2A	0% (0–0)	0% (0)	0% (0–0)[d]	3% (2)	0% (0–0)	0% (0)	0% (0–0)	7% (4)	0% (0–0)	0% (0)	0% (0–0)	5% (2)
APC	0% (0–0)	0% (0)	4% (0–56)[e]	29% (6)	0% (0–0)	0% (0)	0% (0–2)[f]	9% (4)	0% (0–0)	0% (0)	0% (0–46)[e]	20% (4)
CDH1	0% (0–0)	0% (0)	0% (0–0)[d]	3% (2)	0% (0–0)	0% (0)	0% (0–0)[d]	1% (1)	0% (0–0)	0% (0)	0% (0–0)[e]	2% (1)
CMI	7 (6–22)	18 (7)	122 (72–208)[e]	150 (15)	31 (13–78)[g]	54 (21)	59 (31–90)[f]	91 (20)	13 (7–31)	31 (9)	89 (43–184)[e]	126 (13)

SE, standard error; CMI, cumulative methylation index.
[a]Sporadic normal = reduction mammoplasty.
[b]Hereditary normal = prophylactic mastectomy.
[c]IQR , Interquartile range: 25th–75th percentile.
[d]Median for tumor is significantly different from median for normal tissue ($P \leq 0.05$; Mann–Whitney).
[e]Median for tumor is significantly different from median for normal tissue ($P \leq 0.005$; Mann–Whitney).
[f]Median for hereditary is significantly different from sporadic equivalent ($P \leq 0.005$; Mann–Whitney).
[g]Median for hereditary is significantly different from sporadic equivalent ($P \leq 0.05$; Mann–Whitney).

remained significant in all instances ($P = 0.02$, $P = 0.002$, $P = 0.03$, respectively).

discussion

In this study, we showed in univariate and multivariate analysis that the cumulative amount of methylation detected by QM-MSP in a gene panel is significantly correlated to the presence of breast malignancy. We demonstrated that the extent of methylation detected was significantly higher in lymph node-positive compared with lymph node-negative tumors, indicating that our methylation panel is associated not only with the presence of malignancy but also with the metastatic potential of breast tumors.

Interestingly, overall lower methylation was found in BRCA1-associated cancers, presumably resulting in a lower predictive value of this panel for BRCA1-associated tumors than for sporadic tumors. Nevertheless, BRCA1-related cancers still showed a much higher extent of methylation compared with normal tissue. We anticipate that by adding other genes (such as GSTP1 of which methylation has been shown to be an early event in breast carcinogenesis [13]) to our panel, the differences will even be larger. The panel thus holds promise for the early detection of breast cancer in women at high risk, but its actual predictive value needs to be investigated in prospectively collected nipple fluid from high-risk women.

Our results do not agree with those reported by Esteller et al. [25], where non-quantitative MSP found overall promoter methylation frequencies of BRCA1, CDH1, CDKN2A, GSTP1 and RARB, to be similar in 162 hereditary and 106 sporadic breast carcinomas. Since the degree of promoter methylation is closely related to gene expression, the importance of using a quantitative approach like QM-MSP to assess methylation gains credence.

The remarkable correlation between ER negativity and a lower extent of methylation seems to be confined to BRCA1-associated tumors. The question arises if the low methylation values in these tumors are primarily due to the presence of a germline BRCA1 mutation, ER loss or both. Possibly, the four ER-positive tumors in BRCA1 mutation carriers concern sporadic tumors in BRCA1 germline mutation carriers (an event with a chance of 1:9 in the Netherlands) and therefore show a higher extent of methylation than the 'true' BRCA1-associated tumors. This hypothesis would favor a role for BRCA1 mutations in the development of methylation defects, regarding ER negativity as a BRCA1-associated phenomenon. Our subgroup analysis indicated that the lower extent of methylation in BRCA1-associated tumors was independent of ER status, as well as of lymph node status and age.

But why then are lower levels of methylation observed in BRCA1-associated breast cancer? It is known that somatic genetic changes are more frequent in BRCA1-associated tumors compared with sporadic tumors [26,27] due to overall genetic instability caused by functional inactivation of BRCA1. Possibly, BRCA1-associated carcinogenesis is mainly driven by oncogenic mutations, with only a secondary role for methylation inactivating tumor suppressor genes. Further

studies are needed to assess whether BRCA1 loss affects *de novo* methylation patterns in pre-cancerous cells.

Age-related methylation has been shown to be widespread and one of the earliest changes marking the cancer risk, progressively increasing during, e.g. colorectal carcinogenesis [28]. Although a similar age-related increase in methylation in breast tissue has been presumed, the age effect has never been directly shown in breast tissue [29]. Hence, to the best of our knowledge, we are the first to clearly show the correlation between aging and methylation in the breast.

In compliance with this age effect, it would have been ideal to age match the sporadic and BRCA1-associated tumors. However, the amount of available archive material for BRCA1 germline mutation carriers is a limiting factor and selecting sporadic carcinomas occurring at a younger age might introduce a bias, since these tumors might be the first symptom of a hereditary predisposition (BRCA1 germline mutation). We believe that adjusting for age in the regression analysis is a good alternative that shows that the difference between methylation in BRCA1-associated and sporadic tumors is not an age effect.

In summary, the extent of methylation in a commonly used gene panel is lower in BRCA1-associated than in sporadic breast tumors. These results imply that the role of methylation in BRCA1-associated carcinogenesis might be less significant than in sporadic tumors. Further studies are needed to elucidate the mechanisms underlying the differences in epigenetic makeup between BRCA1-associated and sporadic breast cancers.

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Conflict of Interest: QM-MSP has been licensed by SS and MJF. SS, MJF and PvD have a consulting relationship with Oncomethylome Sciences. No conflict of interest for other authors.

references

1. Bray F, McCarron P, Parkin DM. The changing global patterns of female breast cancer incidence and mortality. Breast Cancer Res 2004; 6: 229–239.
2. Ford D, Easton DF, Stratton M et al. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. Am J Hum Genet 1998; 62: 676–689.
3. Struewing JP, Hartge P, Wacholder S et al. The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. N Engl J Med 1997; 336: 1401–1408.
4. Brekelmans CTM, Seynaeve C, Bartels CCM et al. Effectiveness of breast cancer surveillance in BRCA1/2 gene mutation carriers and women with high familial risk. J Clin Oncol 2001; 19: 924–930.
5. Kriege M, Brekelmans CT, Obdeijn IM et al. Factors affecting sensitivity and specificity of screening mammography and MRI in women with an inherited risk for breast cancer. Breast Cancer Res Treat 2006; 100: 109–119.
6. Steenman MJ, Rainier S, Dobry CJ et al. Loss of imprinting of IGF2 is linked to reduced expression and abnormal methylation of H19 in Wilms' tumour. Nat Genet 1994; 7: 433–439.
7. Esteller M. Relevance of DNA methylation in the management of cancer. Lancet Oncol 2003; 4: 351–358.
8. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003; 349: 2042–2054.
9. Ehrlich M. DNA methylation in cancer: too much, but also too little. Oncogene 2002; 21: 5400–5413.
10. Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet 2000; 16: 168–174.
11. Knudson AG. Two genetic hits (more or less) to cancer. Nat Rev Cancer 2001; 1: 157–162.
12. van Diest PJ. Ductal carcinoma in situ in breast carcinogenesis. J Pathol 1999; 187: 383–384.
13. Lee JS. GSTP1 promoter hypermethylation is an early event in breast carcinogenesis. Virchows Arch 2007; 450: 637–642.
14. Deshmane VH, Fentiman IS, Hart IR. Early detection of malignant change in breast cells. Lancet 2001; 358: 507.
15. Fackler MJ, Malone K, Zhang Z et al. Quantitative multiplex methylation-specific PCR analysis doubles detection of tumor cells in breast ductal fluid. Clin Cancer Res 2006; 12: 3306–3310.
16. Fackler MJ, McVeigh M, Mehrotra J et al. Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. Cancer Res 2004; 64: 4442–4452.
17. Trinh BN, Long TI, Nickel AE et al. DNA methyltransferase deficiency modifies cancer susceptibility in mice lacking DNA mismatch repair. Mol Cell Biol 2002; 22: 2906–2917.
18. Ahuja N, Mohan AL, Li Q et al. Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res 1997; 57: 3370–3374.
19. Caldeira JR, Prando EC, Quevedo FC et al. CDH1 promoter hypermethylation and E-cadherin protein expression in infiltrating breast cancer. BMC Cancer 2006; 6: 48.
20. Archey WB, McEachern KA, Robson M et al. Increased CpG methylation of the estrogen receptor gene in BRCA1-linked estrogen receptor-negative breast cancers. Oncogene 2002; 21: 7034–7041.
21. van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. Br Med J 2002; 325: 648–651.
22. van Diest PJ, Baak JP, Matze-Cok P et al. Reproducibility of mitosis counting in 2,469 breast cancer specimens: results from the multicenter morphometric mammary carcinoma project. Hum Pathol 1992; 23: 603–607.
23. Haybittle JL, Blamey RW, Elston CW et al. A prognostic index in primary breast cancer. Br J Cancer 1982; 45: 361–366.
24. Korsching E, Packeisen J, Agelopoulos K et al. Cytogenetic alterations and cytokeratin expression patterns in breast cancer: integrating a new model of breast differentiation into cytogenetic pathways of breast carcinogenesis. Lab Invest 2002; 82: 1525–1533.
25. Esteller M, Fraga MF, Guo M et al. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet 2001; 10: 3001–3007.
26. Tirkkonen M, Johannsson O, Agnarsson BA et al. Distinct somatic genetic changes associated with tumor progression in carriers of BRCA1 and BRCA2 germ-line mutations. Cancer Res 1997; 57: 1222–1227.
27. Jonsson G, Naylor TL, Vallon-Christersson J et al. Distinct genomic profiles in hereditary breast tumors identified by array-based comparative genomic hybridization. Cancer Res 2005; 65: 7612–7621.
28. Ahuja N, Li Q, Mohan AL et al. Aging and DNA methylation in colorectal mucosa and cancer. Cancer Res 1998; 58: 5489–5494.
29. Widschwendter M, Jones PA. DNA methylation and breast carcinogenesis. Oncogene 2002; 21: 5462–5482.