Association of aberrant DNA methylation with clinicopathological features in breast cancer

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Abstract. Aberrant DNA methylation is responsible for the epigenetic silencing of genes associated with tumourigenesis and progression of cancer. In this study, we assessed the methylation status of eight genes in 49 snap-frozen primary breast tumours. Epigenetic alterations of 8 genes were analysed with methylation-specific polymerase chain reaction (MS-PCR) (DCR1, DAPK1, RASSF1A and DCR2) or methylation-sensitive high-resolution melting analysis (MS-HRM) (APC, MGMT, GSTP1 and PTEN). MS-HRM performance was validated by bisulfite pyrosequencing regarding the methylation levels of MGMT. Promoter methylation was observed in APC 54.34%, 40.4% DCR1, 37.5% DAPK1, 33.3% RASSF1A, 22.44% MGMT, 16.6% GSTP1, 6% PTEN and 0% DCR2 promoters, respectively. Interestingly, 37 out of 49 cases (75.5%) displayed aberrant promoter methylation in at least one gene. An association of MGMT promoter methylation with age and tumour grade was recorded. Moreover, a correlation with advanced T-category was elicited for GSTP1, RASSF1 and DAPK1 promoter methylation. Finally, concurrent methylation of several genes showed a marginal statistical relationship with N-category. We conclude that APC, DCR1, DAPK1 and RASSF1A promoter methylation represents a common event in breast cancer tumourigenesis. Our results suggest that GSTP1, RASSF1, DAPK1 and MGMT may be implicated in the acquisition of a more aggressive phenotype in breast cancer.

Introduction

Breast cancer, the most common malignancy among women, is a clinically and morphologically heterogeneous disease (1). Cancer progression involves a stepwise accumulation of genetic alterations such as point mutations, deletions, gene amplifications causing either oncogene activation or tumour suppressor loss (2). Moreover, it has been shown that gene expression pattern is also controlled by epigenetic modifications including DNA methylation and histone acetylation which contribute to tumourigenesis. The best studied modification in cancer is DNA methylation which occurs on cytosine residues of CpG dinucleotides in gene promoters or first exons where they form the so-called CpG islands (3). Aberrant DNA methylation in gene promoters leads to the formation of a repressive chromatin structure inhibiting the access of appropriate transcription factors to certain binding sites, with consequent loss of function of genes involved in tumourigenesis, such as tumour suppressor and DNA repair genes, cell cycle regulators and transcription factors (4,5). Despite intensive research, the molecular pathogenesis of breast cancer has not been fully elucidated. In recent years, the role of DNA methylation in the progression of breast cancer has been the subject of several investigations yielding controversial results (6-8). Nevertheless, important questions such as the stage of occurrence of methylation changes, whether they constitute independent events or occur concurrently remain unanswered.

In the present investigation two different methodologies, namely methylation-specific polymerase chain reaction (MS-PCR; MSP) which uses two specific sets of primers for methylated/unmethylated templates and methylation-sensitive high-resolution melting (MS-HRM) analysis which detects the sequence-dependent melting profile of an amplicon, were applied for the study of 8 genes that potentially contribute to the development of breast cancer. Furthermore, in order to validate results obtained by the MS-HRM method, pyrosequencing was also used for the quantitative assessment of MGMT gene methylation status (9).

RASSF1A (Ras association domain family 1) is a tumour suppressor gene that functions as a pro-apoptotic effector of Ras-mediated signal transduction pathways. It has been suggested that RASSF1A protein functions as a scaffolding protein for the assembly of proteins regulating biological processes such as cell cycle, apoptosis and genomic stability (10). MGMT (O^6-methylguanine-DNA methyltransferase) encodes a DNA repair enzyme which dissociates alkyl adducts

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from the O^6 position of guanine providing a significant defence mechanism of the cell against tumourigenesis (11). APC (Adenomatous polyposis coli) is a tumour suppressor gene with several cellular functions. It has a prominent role in Wnt signal transduction pathway, in intercellular adhesion, in cell cycle regulation and apoptosis (12). DAPK1 (death-associated protein kinase-1), a pro-apoptotic serine/threonine protein kinase gene is also a positive mediator of γ-interferon (IFN-γ) induced programmed cell death (13). GSTP1 (glutathione S-transferase pi1) is a gene with important role in detoxification of xenobiotics, carcinogens, pesticides and several environmental pollutants. It is involved in MAPK signal transduction pathway associated with cell survival and death signalling (14). PTEN (phosphatase and tensin homologue) is a tumour suppressor gene with a dual role as lipid and protein phosphatase regulating PI3K/AKT signalling cascade through its target PIP3 (15). DCR1 and DCR2 (decoy receptor 1 and 2) are genes encoding membrane receptors which can bind TRAIL causing inhibition of TRAIL apoptotic pathway (16).

The aim of this study was to examine 49 cases of breast carcinomas regarding the methylation status of the promoters of the above 8 different genes associated with various cellular functions such as signalling, apoptosis, DNA repair and detoxification, that potentially contribute to the development of breast cancer. The results were correlated with clinicopathological parameters in an effort to delineate their potential role as biomarkers in breast tumourigenesis.

Materials and methods

Patients. In the present study a total of 49 cases with invasive breast cancer (median age 63, age range 38-88) were examined. Clinicopathological data were available for 45 patients, among whom 34 cases were classified as ductal carcinomas, 9 as lobular carcinomas and 2 as mixed carcinomas, diagnosed and treated at the Hippocration Hospital of Athens, Greece. Informed consent was obtained from all patients and the study was approved by the University of Athens Medical School Ethics Committee. According to TNM classification for breast cancer (17), the distribution into T- and N-categories was as follows: T1 (15 cases), T2 (21 cases), T3 (9 cases), N0 (24 cases), N1 (8 cases), N2 (7 cases) and N3 (6 cases). As far as TNM stage grouping is concerned, cases were distributed as follows: T1N0Mx (11 cases), T1N1Mx (2 cases), T1N2Mx (2 cases), T1N3Mx (1 case), T2N0Mx (11 cases), T2N1Mx (5 cases), T2N2Mx (4 cases), T2N3Mx (1 case), T3N0Mx (3 cases), T3N1Mx (1 case), T3N2Mx (1 case) and T3N3Mx (5 cases). All specimens were assigned a histological grade (grade II, 15; grade III, 21 and grades II-III, 6) based on the degree of tumour differentiation (well, moderate and poorly differentiated, respectively) according to WHO histological classification of tumours (18). The ER/PR status was also assessed, 69% of the cases being ER positive and 60% were PR positive.

Genomic DNA isolation. DNA extraction from 49 snap-frozen tissues, was performed by standard protocols using proteinase K followed by phenol/chloroform (1:1) extraction, ice-cold ethanol precipitation. The DNA concentration was quantitated using the Picodrop Microliter spectrophotometer.

Bisulfite treatment and methylation-specific polymerase chain reaction (MSP). DNA isolated from tumour specimens (0.5-5 μg) was treated with sodium bisulfite using the EZ DNA methylation kit according to the manufacturer's protocol (Zymo Research, Orange, CA, USA).

We detected the methylation status of DAPK1, RASSF1A, DCR1 and DCR2 using MSP. MSP was performed in a 25 μl volume PCR reaction using ~1-2 μl-bisulfite-modified DNA template and Taq DNA polymerase (Titanium, BD Biosciences, USA). After an initial denaturation step for 2 min at 94˚C, 40 cycles at 94˚C for 30 sec, at different temperatures depending on the primer pairs (Table I) for 40 sec and at 72˚C for 40 sec and 7 min at 72˚C, were carried out in a Techne Progene Thermal Cycler. PCR products were visualised by electrophoresis on a 4% agarose gel.

Methylation-sensitive high-resolution melting analysis (MS-HRM). In order to detect the promoter methylation status of MGMT, APC, PTEN and GSTP1 genes we used a real-time polymerase chain reaction (PCR) approach followed by high resolution melting curve analysis (HRM), considered as a rapid, highly sensitive and efficient method displaying the sequence-dependent melting profile of an amplicon. PCR and HRM analysis were consecutively performed on a LightCycler 480 (Roche Diagnostics GmbH, Germany) in one single run, and all samples were analysed in duplicate. Each reaction mixture contained ~1-2 μl of bisulfite-treated DNA, 200 nmol/μl of each primer, 10 μl of LightCycler 480 High Resolution Melting Master (Roche), 3.5 mM $MgCl_2$ and PCR-grade water adjusted to a total volume of 20 μl. The conditions used in the LightCycler 480 were an initial cycle at 95˚C for 15 min, followed by 50 cycles of 95˚C for 10 sec, annealing temperatures depending on the respective primer pairs (Table I) for 15 sec, 72˚C for 7 sec and one cycle at 95˚C for 1 min. HRM was performed from 65 to 95˚C with a temperature increase at 0.10˚C/sec.

Bisulfite pyrosequencing. Pyrosequencing is a novel sensitive technique for accurate and quantitative analysis of DNA sequences and their methylation status. PCR reaction was performed in a 50-μl volume reaction with ~100 ng template of bisulfite-modified DNA. PCR conditions for MGMT gene were: 2 min at 95˚C; 20 sec at 95˚C, 30 sec at 57˚C (Table I), 20 sec at 72˚C for 50 cycles; 7 min at 72˚C. The sequences of primers are shown in Table I. The methylation status of the MGMT gene promoter was detected by pyrosequencing analysis of the PCR product without any further purification using the Q24 Qiagen Pyrosequencer (Qiagen, Biotage). For the pyrosequencing reaction, 40 μl of biotinylated PCR product was used for analysis with the PyroMark Gold Q24 Reagents kit according to the manufacturer's protocol.

Statistical analysis. Statistical analysis was performed for 45 cases with available clinicopathological data. The respective correlations between gene promoter methylation status and age were examined using the Mann-Whitney U test. Associations of gene promoter methylation status with clinicopathological characteristics, such as T- and N-category of TNM classification were tested with the Fisher's exact test and the Kruskal-Wallis ANOVA, respectively, as appropriate.

Table I. Primer sequences used for MSP, MS-HRM and pyrosequencing.

Gene name	Primer sequence 5'-3'	Method
APC	F: AAGTAGTTGTGTAATTCGTTGGAT R: CACCTCCATTCTATCTCCAATA	MS-HRM
MGMT	F: GCGTTTCGGATATGTTGGGATAGT R: AACGACCCAAACACTCACCAAA	MS-HRM
GSTP1	F: GTGAAGCGGGTGTGTAAGTTT R: TAAACAAACAACAAAAAAAAAACC	MS-HRM
PTEN	F: TCGGTTGGGTTTTTGGGTAGAGG R: CGCAAACTCTACTAAACATACCCAATAT	MS-HRM
MGMT	F: GTTTYGGATATGTTGGGATAGTT R: ACRCCTACAAAACCACTCRA Seq: CCAAACACTCACCAAAT	Pyrosequencing
DCR1	F unmethylated: GAATTTTTTTATGTGTATGAATTTAGTTAAT R unmethylated: CCATCAAACAACCAAAACA F methylated: TTACGCGTACGAATTTAGTTAAC R methylated: ATCAACGACCGACCGAAACG	MSP
DCR2	F unmethylated: TTGGGGATAAAGTGTTTTGATT R unmethylated: AAACCAACAACAAAACCACA F methylated: GGGATAAAGCGTTTCGATC R methylated: CGACAACAAAACCGCG	MSP
DAPK1	F unmethylated: GGAGGATAGTTGGATTGAGTTAATGTT R unmethylated: CAAATCCCTCCCAAACACCAA F methylated: GGATAGTCGGATCGAGTTAACGTC R methylated: CCCTCCCAAACGCCGA	MSP
RASSF1A	F unmethylated: TTTGGTTGGAGTGTGTTAATGTG R unmethylated: CAAACCCCACAAACTAAAAACAA F methylated: GTGTTAACGCGTTGCGTATC R methylated: AACCCCGCGAACTAAAAACGA	MSP

MSP, methylation-specific polymerase chain reaction; MS-HRM, Methylation-sensitive high-resolution melting analysis.

Statistical calculations were performed using the statistical package STATA SE/9.0 for Windows. All results with a two-sided p-value <0.05 were considered significant.

Results

DCR1, DAPK1, RASSF1A and DCR2 methylation analysis using MSP. In order to evaluate the methylation status of DCR1, DAPK1, RASSF1A and DCR2 gene promoters MSP analysis was performed. Results are shown in Fig. 1. Aberrant methylation of DCR1 was observed in 19 out of 47 breast cancer patients (40.4%), and more specifically in 11 out of 32 (34.6%) invasive ductal carcinomas and 5 out of 8 (62.5%) invasive lobular carcinomas. Hypermethylation of DAPK1 was found in 18 out of 48 specimens (37.5%) and as for RASSF1A, promoter methylation occurred at a frequency of 33.3% (16 out of 48 cases). Characteristically, 13 out of 33 invasive ductal carcinomas (39.4%) and 4 out of 8 invasive lobular carcinomas (50%) were DAPK1 methylated whereas 13 out of 33 invasive ductal carcinomas (39.4%) and 3 out of 8 invasive lobular carcinomas (37.5%) were RASSF1A methylated. DCR2 methylation was not observed (none of 49 patients). Representative methylated and unmethylated PCR products for all the examined genes, as assessed by MSP assay, were verified by sequencing.

MS-HRM analysis. In order to determine the relative methylated status of APC, MGMT, GSTP1 and PTEN promoters we applied a sensitive semi-quantitative method, namely MS-HRM analysis. Aberrant methylation of APC was very frequent, observed in 25 out of 46 (54.34%) breast cancer specimens, in particular 17 out of 31 (54.83%) invasive ductal carcinomas and 6 out of 8 (75%) invasive lobular carcinomas. The majority of the cases displayed evident methylated peaks indicating the presence of high level methylation. However, a proportion of the samples showed small methylated peaks consistent with low levels of methylation which could be attributed to the existence of a higher proportion of unmethylated than methylated alleles in the sample or to the presence of alleles with low percentage methylation in all examined CpGs

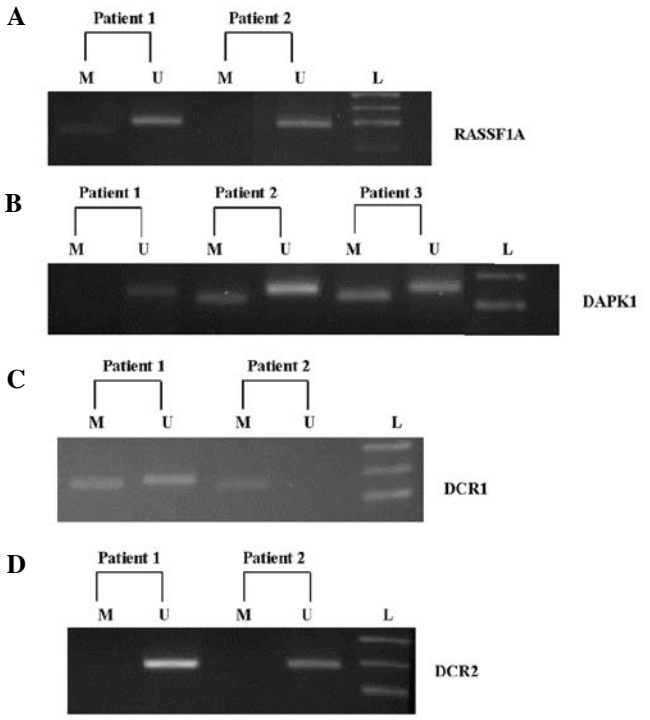

Figure 1. Representative results of MSP analysis for (A) RASSF1A, (B) DAPK1, (C) DCR1 and (D) DCR2 in primary breast cancer patients. Lanes M and U correspond to methylated and unmethylated bisulfite-treated DNA respectively and L to a 1-kb ladder as molecular weight marker. PCR products were visualised with ethidium bromide after electrophoresis on a 4% agarose gel.

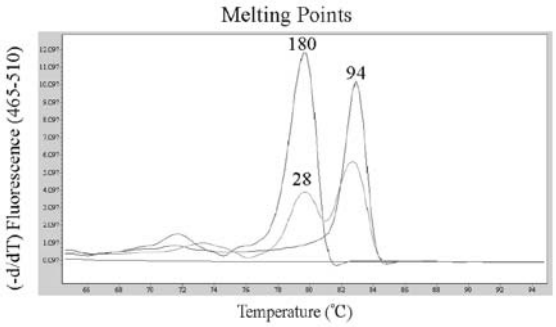

Figure 2. APC promoter methylation profile (T_m plot) observed with MS-HRM. Unmethylated sample 180 melts earlier than methylated samples due to DNA bisulfite modification where unmethylated cytosines replaced by thymines. Sample 94 was a fully methylated case. Sample 28 was heterogeneously methylated showing a methylation profile with a mixture of methylated and unmethylated peaks.

(39). Representative results are shown in Fig. 2. Promoter methylation of MGMT and GSTP1 was moderate observed in 11/49 cases (22.44%) and 8/48 (16.6%) cases, respectively. In detail, 8 out of 34 invasive ductal carcinomas (23.5%) and 1 out of 9 invasive lobular carcinomas (11.1%) were MGMT-methylated whereas 7 out of 33 invasive ductal carcinomas (21.21%) and 1 out of 9 invasive lobular carcinomas (11.1%) were GSTP1 methylated. Methylation of PTEN was quite low, reaching 6% (3/49) [2 out of 34 invasive ductal carcinomas (5.88%) and 1 out of 9 invasive lobular carcinomas, (11.1%)]. After MS-HRM, random PCR products for every examined gene were further analysed by sequencing in order to check

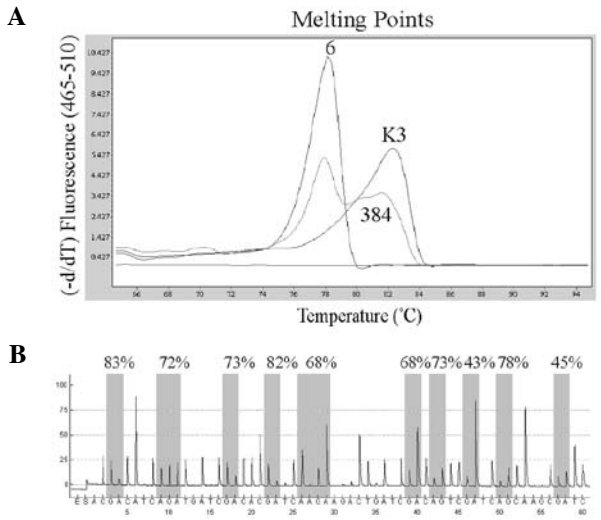

Figure 3. Comparison of MS-HRM and pyrosequencing. (A) MS-HRM analysis of a representative case, sample 384, showed high levels of methylation in relation to a highly methylated control (K3). These results were in accordance with the MGMT pyrogram (B) of the same sample (384). This tumor sample was highly methylated in all 10 CpG positions as analysed by pyrosequencing. In the pyrogram peaks highlighted by light gray shading were used in order to calculate the methylation ratio at each CpG position. At the top of each highlighted area it is shown the observed methylation percentage of each CpG. Nucleotides 13, 31, 35 and 55 are control peaks to evaluate an incomplete bisulfite conversion.

the specificity of the assay. It is worthy of note that methylated as well as unmethylated sequences were detected in the majority of the samples by both applied methods.

Concordance between MS-HRM and pyrosequencing. In order to validate MS-HRM analysis, results obtained for MGMT promoter methylation in 34 samples were compared with pyrosequencing data. Using bisulfite pyrosequencing we analysed and quantified, calculating an average level of methylation for each case, the methylation status of 10 CpGs in the MGMT promoter region. Four cases were methylated in all analysed CpGs showing average levels of methylation ranging from 25-95% as assessed by pyrosequencing. Three of these cases displaying high average level of methylation (60-95%) were also found heavily methylated by HRM analyses. The last case showed a lower methylated peak by MS-HRM in parallel with lower levels of average methylation (25%) across all examined CpGs by pyrosequencing. Two other cases were methylated in 9 and 8 CpG positions with an average methylation level of 15% displaying a similar result by HRM as indicated by low methylated peaks. Moreover, there were two samples displaying methylation in only 3 and 1 of the examined CpG sites respectively, showing average methylation levels of 35% in these specific locations. We observed very low peaks by HRM in these specific samples. Low average levels of methylation by pyrosequencing are due either to the presence of many more unmethylated than methylated alleles in the sample or to low levels of methylation of all the analysed CpGs. Overall, a high concordance between results obtained by semi-quantitative MS-HRM and those obtained by quantitative pyrosequencing was seen. Representative results of the MGMT gene are shown in Fig. 3.

Table II. Distribution of methylated genes in 49 primary breast tumor cases.

	MGMT	PTEN	APC	GSTP1	RASSF1A	DAPK1	DCR1	DCR2
6	U	M	U	U	M	M	U	U
18	M	U	U	U	M	M	U	U
20	U	U	U	U	M	M	M	U
28	U	U	M	M	U	M	U	U
30	U	U	M	U	M	M	M	U
34	U	U	U	U	U	U	U	U
46	U	U	M	U	M	M	U	U
50	M	U	M	U	M	M	M	U
54	U	U	M	U	U	M	M	U
62	U	U	U	M	M	M	U	U
64	U	U	M	U	M	U	U	U
68	U	U	M	M	M	M	M	U
70	U	U	U	U	U	U	U	U
72	U	U	U	U	U	U	M	U
74	M	U	U	U	U	U	U	U
76	M	U	M	U	U	M	-	U
78	M	U	M	U	U	U	U	U
80	U	U	M	U	U	U	U	U
82	U	U	-	M	U	U	U	U
84	U	U	U	U	U	U	U	U
86	U	U	U	U	M	M	M	U
88	U	U	M	U	U	U	M	U
94	U	U	M	U	M	M	U	U
98	U	U	U	M	M	U	M	U
106	U	U	M	M	M	U	U	U
108	U	U	U	U	U	U	M	U
116	U	U	U	U	U	U	U	U
118	U	M	M	U	U	M	M	U
128	U	U	U	U	U	U	U	U
164	U	U	U	U	U	U	U	U
166	U	U	U	U	U	U	U	U
168	M	U	M	U	-	U	M	U
178	U	U	U	U	U	U	U	U
180	U	U	M	U	U	U	U	U
184	U	U	M	U	U	M	U	U
188	M	U	M	U	U	U	M	U
196	U	U	U	U	U	U	U	U
198	M	U	-	M	U	U	U	U
200	U	U	U	U	U	U	U	U
214	U	U	U	U	U	U	U	U
240	U	U	-	-	U	-	-	-
258	U	U	M	M	U	U	U	U
308	U	U	M	U	M	M	M	U
310	U	U	M	U	M	M	M	U
318	U	U	M	U	M	M	M	U
364	U	M	U	U	U	U	U	U
372	U	U	-	U	U	U	M	U
382	M	U	U	U	U	U	M	U
384	M	U	U	U	U	U	M	U

Methylation profile of 49 primary breast carcinomas. Each column represents one gene and each row is a tumor sample. M, methylated genes U, unmethylated genes; -, samples not being amplified by MSP or MS-HRM.

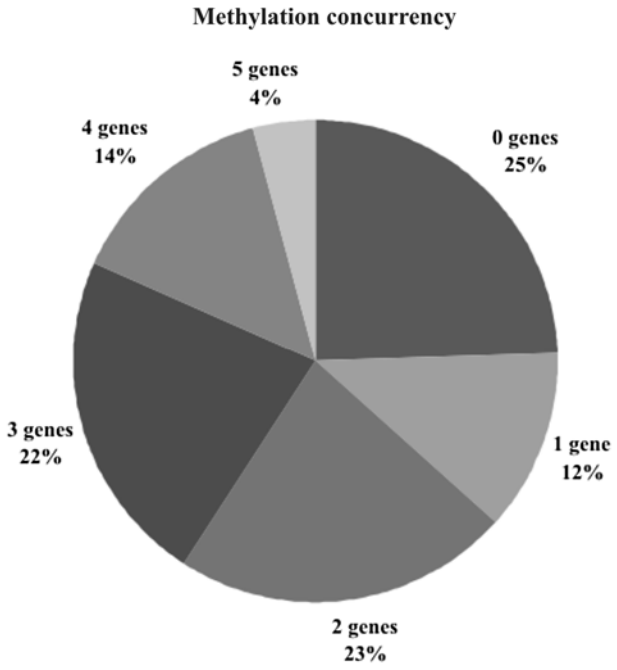

Figure 4. Percentages of concurrent aberrant methylated gene promoters in 49 primary breast tumors.

Concurrent methylated genes. In the present study 7 of the 8 analysed genes showed promoter methylation in at least one of the examined cases. Results are summarised in Table II. In particular, 22.44% (11/49) of the samples displayed concurrent methylation of 2 genes and 12.24% (6/49) were methylated in one gene only. Simultaneous methylation of 3 genes was identified in 11 out of 49 cases (22.44%). Concomitant aberrant methylation in promoter regions of 4 or 5 genes was detected in 14.28% (7/49) and 4.08% (2/49) of the cases, respectively. None of the analysed samples displayed methylation in more than five different promoters (Fig. 4). Finally, twelve cases displayed no methylation at all.

Associations between methylated genes and clinicopathological features. MGMT methylation was more often observed in older patients (Mann-Whitney U test, p=0.0222), whereas there was a marginal correlation of MGMT aberrant methylation with advanced tumour grade (Fisher's exact test, p=0.067). GSTP1 promoter methylation seems to be marginally correlated with advanced T-stage (Fisher's exact test, p=0.057). Moreover, tumours with advanced T-category showed a higher frequency of RASSF1A and DAPK1 methylation (Fisher's exact test, p=0.033 and p=0.078, respectively), the latter relationship being of marginal statistical significance. Furthermore promoter methylation of RASSF1A and DCR1 showed a marginal correlation with PR status (Fisher's exact test, p=0.056 and p=0.051, respectively). The overall percentage of concurrent methylation status taking into account all 7 methylated genes ranged from 0 to 62.5% (median 31.25%). A trend for a positive correlation between higher overall percentage of concurrent methylation status and N-category (Kruskal Wallis ANOVA, p=0.0744) was noted.

On the other hand, the presence of methylation and the expression of ER, c-ERB-b2, p53 and Ki-67 were not related (p>0.10). All the associations of each gene methylation status with clinicopathological characteristics are presented in Table III.

Discussion

The delineation of a specific DNA methylation signature in breast tumourigenesis could impact the development of new approaches for diagnosis and clinical management. Studies reporting detection of aberrant DNA methylation of RASSF1A, APC, GSTP1 and DAPK1 genes in serum or plasma of breast cancer patients suggest their possible value as biomarkers for monitoring carcinogenesis (19). Moreover, existing demethylating agents and DNA methylation inhibitors have come to the forefront for breast cancer therapy (20). Lastly, it has been argued that methylation analyses could prove to be useful tools towards a molecular classification of breast tumour histological subtypes.

Aberrant promoter DNA methylation has been examined with various methodologies, including COBRA, SSCP, MSP and sequencing (21). MSP is currently the most widely used method due to its claimed efficiency in heterogeneous cancer cell populations (19). On the other hand it has limitations since it is time-consuming, more expensive and not quantitative in comparison with MS-HRM (21). MS-HRM is a novel approach to identify aberrant methylation of gene promoter regions using sequence-dependent melting profiles of each amplicon (22). It has been considered as the most rapid and sensitive in-tube method capable of detecting even 0.1-1% of DNA methylation in an unmethylated background, minimizing possible sample contamination, and requiring only low amounts of DNA template (9,21). Furthermore, MS-HRM is a semi-quantitative method that has been claimed to distinguish homogeneous from heterogeneous methylation (9). In order to validate the sensitivity of the newly established MS-HRM analysis for methylation detection we performed pyrosequencing analysis for MGMT gene promoter methylation status in most of the cases. Bisulfite pyrosequencing analyses short genomic regions (<100 bp), therefore, it is suitable for methylation studies of degraded genomic material extracted from formalin-fixed paraffin embedded tissues (FFPE) (9). It is considered as a very sensitive (detects 5% levels of DNA methylation in an unmethylated background) and quantitative approach for the examination of heterogeneously methylated promoters as well as capable of detecting the methylation levels of individual CpGs within an amplicon (23). For these reasons it has been described as a complementary method of MS-HRM in DNA methylation studies (9). Furthermore, it comprises control positions for the efficiency of bisulfite treatment. Even though pyrosequencing is a very sensitive technique in methylation analyses it is of higher cost and more labour-intensive than MS-HRM, leading to the use of the latter as a screening method in order to discriminate between methylated and unmethylated samples followed by pyrosequencing of only the methylated ones (9).

In the present study we used two different approaches, namely MSP or MS-HRM analysis in order to detect the methylation status of normally unmethylated genes involved in various cellular functions: three tumour suppressor genes (APC, RASSF1A and PTEN), three genes related to apoptosis

Table III. Association of gene methylation with clinicopathological characteristics.

	MGMT n (%)	GSTP1 n (%)	PTEN n (%)	APC n (%)	RASSF1A n (%)	DAPK1 n (%)	DCR1 n (%)
Histology							
Lobular	1 (11.1)	1 (11.1)	1 (11.1)	6 (75)	3 (37.5)	4 (50)	5 (62.5)
Ductal	8 (23.5)	7 (21.2)	2 (5.88)	17 (54.8)	13 (39.4)	13 (39.4)	11 (34.6)
Lobular+ductal				1 (50)		1 (50)	
Grade							
II	0 (0)	5 (33.3)	2 (13.3)	6 (46.15)	4 (25)	6 (37.5)	4 (25)
III	6 (28.57) p=0.067	3 (15)	0 (0)	15 (60)	11 (44)	11 (44)	11 (45.8)
ER							
-	4 (30.76)	1 (7.69)	1 (8)	10 (62.5)	3 (25)	6 (50)	7 (58.3)
+	5 (17.2)	6 (20.7)	2 (6.8)	13 (54.16)	12 (41.4)	10 (34.5)	8 (28.6) p=0.091
PR							
-	4 (23.52)	2 (12.5)	1 (6)	10 (62.5)	3 (18.7)	7 (43.7)	9 (60)
+	5 (19.23)	5 (19.23)	2 (7.69)	13 (54.16)	13 (50) p=0.056	10 (38.5)	7 (26.9) p=0.051
c-Erb-B2							
-	6 (35.29)	6 (35.29)	1 (5.8)	9 (60)	7 (41.2)	7 (43.7)	6 (35.29)
+	2 (8.3)	1 (4.16)	2 (8.3)	13 (56.52)	8 (33.3)	9 (36)	2 (8.3)
p53							
-	7 (23.3)	5 (16.6)	3 (10)	18 (62.06)	12 (40)	12 (40)	11 (37.9)
+	2 (25)	1 (12.5)	0 (0)	3 (37.5)	2 (28.6)	3 (42.8)	3 (42.8)
Ki-67							
-	3 (33.3)	3 (33.3)	0 (0)	6 (75)	1 (20)	0 (0)	1 (20)
+	5 (16.2)	4 (13.3)	2 (6.45)	16 (53.33)	14 (41.2)	16 (45.7) p=0.071	14 (41.2)
T-category							
T1	3 (20)	4 (25)	1 (6.66)	7 (50)	2 (12.5)	3 (18.7)	5 (31.2)
T2	4 (18.2)	0 (0)	2 (9.52)	13 (61.9)	6 (42.9)	7 (50)	4 (30.8)
T3	2 (22.2)	2 (40) p=0.067	0 (0)	4 (44.4)	3 (75) p=0.033	3 (60) p=0.078	3 (60)

(DCR1, DCR2 and DAPK1), MGMT, a DNA repair gene and GSTP1-glutathione S-transferase. These genes display widespread methylation in solid cancers such as colon, gastric, oesophageal, brain, lung and cervix (24-26). Pyrosequencing analysis was applied in order to validate results obtained by MS-HRM. Furthermore, we searched for any correlations between the methylation status of the examined gene promoters and the available clinicopathological characteristics i.e., age, tumour grade, stage, histological type (ductal, lobular), ER/PR, p53, cERB-b2 and Ki-67 status in 49 primary breast tumours.

The most prominent hypermethylation of the current study was seen in the APC promoter region. We report methylation in 54.34% of the cases, a finding consistent with previous studies demonstrating a great variation in the methylation status of APC ranging from 5 to 57% (25,27). Concerning the two major histological types, APC has been previously reported to be methylated in 38-55% of invasive ductal carcinomas (6,19,28,29) and in 50% of invasive lobular carcinomas (19). In this study, the respective frequencies were 54.83 and 75%. This variation of the observed methylation frequency could be attributed to the reduced sensitivity of MSP- used in most of these studies in comparison with MS-HRM analysis applied in the present study. Aberrant methylation of APC was seen in invasive ductal and lobular carcinomas of all examined pathological grades and stages, possibly reflecting its occurrence as an early event in breast tumourigenesis.

Pronounced methylation was also observed for the DCR1 gene promoter in our cohort at a frequency of 40%. DCR1 methylation patterns have not been extensively examined in breast tumours. In one previous investigation conducted in American breast cancer patients a similar methylation frequency of 33% (30% of invasive ductal and 50% of invasive lobular carcinomas) was recorded (26). The increased frequency of DCR1 promoter methylation highlights its

possible significance for breast carcinogenesis. However, correlations of the methylation status with other clinicopathological features were not attained, apart from a marginal correlation with PR status.

In our cohort DAPK1 promoter methylation was found in 37.5% of the cases (ductal 39.4% and lobular 50%). The frequencies in primary breast tumours reported in the literature fluctuate between 7-50% (19,25,30). A marginally significant statistical correlation of DAPK1 promoter methylation with advanced T-category and PR status was found in our series, disputing a previous report which failed to substantiate such an association (30).

Although RASSF1A promoter methylation has been extensively analysed in breast tumourigenesis, a consensus has not been reached due to the great fluctuation of the results displaying low to very high methylation levels (9-65%). Our findings fall in the middle of the reported range (33.3%) in accordance with several previous studies (19,27,31-33). As far as the two major histological subtypes are concerned, a similar frequency of methylation was detected as previously reported (6,29,34). Interestingly, a statistically-significant relationship between RASSF1A promoter methylation and advanced T-category was found in accordance to Karray-Chouayekh *et al* (35) suggesting its involvement as a late event in breast tumourigenesis. On the other hand, Kioulafa *et al* (33) reported a correlation of RASSF1A promoter methylation with early tumour stages.

The observed incidence of MGMT methylation as assessed by MS-HRM and validated by pyrosequencing was 22%, in contrast to an earlier investigation analysing a smaller cohort with MSP (25) reporting absence of methylation in primary breast tumours. As far as invasive ductal breast carcinomas are concerned, methylation up to 32% of the cases has been reported (28,34,36) in accordance with our findings. Finally, in invasive lobular breast carcinomas, for which there are currently no data, we detected methylation in a subset of the examined cases (11.1%). An interesting finding of our study is the marginal correlation of MGMT aberrant methylation with advanced tumour grade implicating MGMT methylation in the acquisition of a more aggressive phenotype. Another interesting finding of the present investigation is the observed statistical correlation of MGMT methylation and patients' age. Our study analysing Caucasian breast cancer patients supports a previous report showing increased MGMT methylation in ductal carcinomas from elderly Indian breast cancer patients (36). The exact impact of aging in methylation status remains unknown, even though promoter methylation of several genes correlates with age (37). It has been hypothesised that factors such as carcinogens, radiation, reactive oxygen species and the genetic background could contribute to the alteration of methylation patterns during aging. MGMT, a gene associated with such mechanisms could undergo an age-related methylation. We validated the results of MS-HRM analysis concerning the methylation status of MGMT promoter with pyrosequencing analysis in 34 out of 49 tumours. Concordant results were obtained by the two approaches. Using pyrosequencing we were able to separately analyse and quantify the methylation status of 10 CpGs in MGMT promoter region. The 8 methylated cases showed fluctuation in the average methylation ranging between 15-95% and differences in the patterns of methylation across the analysed CpG positions. Differences in the average methylation levels were also monitored by HRM although they could not be assigned to specific CpG sites. This result could be attributed either to the simultaneous analysis of non-cancerous cells in the samples or to the heterogeneity of cell populations present in a tumour regarding the DNA methylation profile. Furthermore, as evidenced clearly by pyrosequencing analysis the possibility of heterogeneously methylated regions is quite common.

GSTP1 promoter methylation frequency remains controversial in breast cancer as well as in invasive ductal carcinomas due to the fluctuation of reported frequencies ranging from 0 to 39% (25,28,34,36,38,39). In the present study 16.6% of breast tumours were found methylated and more specifically 21.21% of invasive ductal carcinomas and 11.1% of invasive lobular carcinomas. A marginal correlation of GSTP1 promoter methylation with advanced T-stage was found in accordance with Arai *et al* (40) suggesting that GSTP1 methylation is more likely a late event in the pathogenesis of breast tumours.

In the present study, PTEN and DCR2 were the genes with the lowest promoter methylation frequency. In breast carcinomas results concerning PTEN promoter methylation are not consistent (34,39). We detected a low incidence of promoter methylation (6%) without any clinicopathological associations. As far as DCR2 is concerned there is still very little information about its implication in breast cancer development (26). Shivapurkar *et al* (26) detected DCR2 methylation in about 60% of primary breast tumours in contrast with our findings suggesting absence of methylation. Possible explanations for these discrepancies include: ethnic variation, and tumour tissue heterogeneity.

The majority (75.51%) of primary breast tumours examined displayed aberrant methylation of at least one gene whereas 63.26 and 40.8% of the cases displayed methylation in more than two or three genes respectively. Concurrent methylation was marginally correlated with N-category. These results are in favour of the hypothesis that simultaneous methylation of cancer-related genes may play a prominent role in the development of breast cancer and could be linked to disease prognosis (41). Should this hypothesis be confirmed, the use of more aggressive therapeutic approaches might be justified in cases with concurrent aberrant methylation.

In conclusion, we observed aberrant methylation of DCR1, DAPK1 and RASSF1A by MSP analysis and detected methylated loci in APC, MGMT, GSTP1 and PTEN promoters by MS-HRM in breast cancer specimens. The results obtained by MS-HRM regarding the MGMT gene were validated by bisulfite pyrosequencing. An association of MGMT promoter methylation with age and tumour grade was recorded. Moreover, a correlation with advanced T-category was found for GSTP1, RASSF1 and DAPK1 promoter methylation. Finally, concurrent methylation of several genes showed a marginal statistical relationship with N-category reflecting the necessity for simultaneous detection of methylation in more than one gene in order to increase the clinical usefulness of methylation analyses for breast tumourigenesis. Further examination of genes displaying high methylation levels in larger cohorts could provide insight for new biomarkers for the early detection and/or disease monitoring, possibly influencing therapeutic decisions.

References

1. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ and Thun MJ: Cancer statistics, 2005. CA Cancer J Clin 55: 10-30, 2005.
2. Hanahan D and Weinberg RA: The hallmarks of cancer. Cell 100: 57-70, 2000.
3. Jovanovic J, Ronneberg JA, Tost J and Kristensen V: The epigenetics of breast cancer. Mol Oncol 4: 242-254, 2010.
4. Esteller M: Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 8: 286-298, 2007.
5. Esteller M: Epigenetics in cancer. New Engl J Med 358: 1148-1159, 2008.
6. Park SY, Kwon HJ, Lee HE, *et al*: Promoter CpG island hypermethylation during breast cancer progression. Virchows Arch 458: 73-84, 2011.
7. Orlando FA and Brown KD: Unraveling breast cancer heterogeneity through transcriptomic and epigenomic analysis. Ann Surg Oncol 16: 2270-2279, 2009.
8. Brooks J, Cairns P and Zeleniuch-Jacquotte A: Promoter methylation and the detection of breast cancer (Review). Cancer Causes Control 20: 1539-1550, 2009.
9. Candiloro IL, Mikeska T and Dobrovic A: Assessing combined methylation-sensitive high resolution melting and pyrosequencing for the analysis of heterogeneous DNA methylation. Epigenetics 6: 500-507, 2011.
10. Donninger H, Vos MD and Clark GJ: The RASSF1A tumor suppressor. J Cell Sci 120: 3163-3172, 2007.
11. Pegg AE: Repair of O (6)-alkylguanine by alkyltransferases. Mut Res 462: 83-100, 2000.
12. Fearnhead NS, Britton MP and Bodmer WF: The ABC of APC. Hum Mol Genet 10: 721-733, 2001.
13. Levy-Strumpf N and Kimchi A: Death associated proteins (DAPs): from gene identification to the analysis of their apoptotic and tumor suppressive functions. Oncogene 17: 3331-3340, 1998.
14. Laborde E: Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death. Cell Death Differ 17: 1373-1380, 2010.
15. Chalhoub N and Baker SJ: PTEN and the PI3-kinase pathway in cancer. Annu Rev Pathol 4: 127-150, 2009.
16. Johnstone RW, Frew AJ and Smyth MJ: The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat Rev Cancer 8: 782-798, 2008.
17. Sobin L, Gospodarowicz MK and Wittekind CH (eds): TNM Classification of Malignant Tumours. 7th edition. Wiley-Blackwell, New York, 2009.
18. Tavassoli FA and Devilee P (eds): Pathology and Genetics of Tumours of the Breast and Female Genital Organs. WHO Classification of Tumours. IARC Press, Lyon, 2003.
19. Dulaimi E, Hillinck J, Ibanez de Caceres I, Al-Saleem T and Cairns P: Tumor suppressor gene promoter hypermethylation in serum of breast cancer patients. Clin Cancer Res 10: 6189-6193, 2004.
20. Dworkin AM, Huang TH and Toland AE: Epigenetic alterations in the breast: implications for breast cancer detection, prognosis and treatment (Review). Semin Cancer Biol 19: 165-171, 2009.
21. Wojdacz TK and Dobrovic A: Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. Nucleic Acids Res 35: e41, 2007.
22. Wojdacz TK and Dobrovic A: Melting curve assays for DNA methylation analysis. Methods Mol Biol 507: 229-240, 2009.
23. Colella S, Shen L, Baggerly KA, Issa JP and Krahe R: Sensitive and quantitative universal pyrosequencing methylation analysis of CpG sites. Biotechniques 35: 146-150, 2003.
24. Kang GH, Lee S, Kim JS and Jung HY: Profile of aberrant CpG island methylation along the multistep pathway of gastric carcinogenesis. Lab Invest 83: 635-641, 2003.
25. Esteller M, Corn PG, Baylin SB and Herman JG: A gene hypermethylation profile of human cancer. Cancer Res 61: 3225-3229, 2001.
26. Shivapurkar N, Toyooka S, Toyooka KO, *et al*: Aberrant methylation of trail decoy receptor genes is frequent in multiple tumor types. Int J Cancer 109: 786-792, 2004.
27. Lewis CM, Cler LR, Bu D, *et al*: Promoter hypermethylation in benign breast epithelium in relation to predicted breast cancer risk. Clin Cancer Res 11: 166-172, 2005.
28. Hoque MO, Prencipe M, Poeta ML, *et al*: Changes in CpG islands promoter methylation patterns during ductal breast carcinoma progression. Cancer Epidemiol Biomarkers Prev 18: 2694-2700, 2009.
29. Cho YH, Yazici H, Wu HC, Terry MB, Gonzalez K, Qu M, Dalay N and Santella RM: Aberrant promoter hypermethylation and genomic hypomethylation in tumor, adjacent normal tissues and blood from breast cancer patients. Anticancer Res 30: 2489-2496, 2010.
30. Lehmann U, Celikkaya G, Hasemeier B, Länger F and Kreipe H: Promoter hypermethylation of the death-associated protein kinase gene in breast cancer is associated with the invasive lobular subtype. Cancer Res 62: 6634-6638, 2002.
31. Agathanggelou A, Honorio S, Macartney DP, *et al*: Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. Oncogene 20: 1509-1518, 2001.
32. Burbee DG, Forgacs E, Zöchbauer-Müller S, *et al*: Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J Natl Cancer Inst 93: 691-699, 2001.
33. Kioulafa M, Kaklamanis L, Mavroudis D, Georgoulias V and Lianidou ES: Prognostic significance of RASSF1A promoter methylation in operable breast cancer. Clin Biochem 42: 970-975, 2009.
34. Muggerud AA, Rønneberg JA, Wärnberg F, *et al*: Frequent aberrant DNA methylation of ABCB1, FOXC1, PPP2R2B and PTEN in ductal carcinoma in situ and early invasive breast cancer. Breast Cancer Res 12: R3, 2010.
35. Karray-Chouayekh S, Trifa F, Khabir A, *et al*: Aberrant methylation of RASSF1A is associated with poor survival in Tunisian breast cancer patients. J Cancer Res Clin Oncol 136: 203-210, 2010.
36. Sharma G, Mirza S, Parshad R, Srivastava A, Gupta SD, Pandya P and Ralhan R: Clinical significance of promoter hypermethylation of DNA repair genes in tumor and serum DNA in invasive ductal breast carcinoma patients. Life Sci 87: 83-91, 2010.
37. Ahuja N and Issa JP: Aging, methylation and cancer. Histol Histopathol 15: 835-842, 2000.
38. Lee JS: GSTP1 promoter hypermethylation is an early event in breast carcinogenesis. Virchows Arch 450: 637-642, 2007.
39. Buyru N, Altinisik J, Ozdemir F, Demokan S and Dalay N: Methylation profiles in breast cancer. Cancer Invest 27: 307-312, 2009.
40. Arai T, Miyoshi Y, Kim SJ, Taguchi T, Tamaki Y and Noguchi S: Association of GSTP1 CpG islands hypermethylation with poor prognosis in human breast cancers. Breast Cancer Res Treat 100: 169-176, 2006.
41. Sharma G, Mirza S, Yang YH, Parshad R, Hazrah P, Datta Gupta S and Ralhan R: Prognostic relevance of promoter hypermethylation of multiple genes in breast cancer patients. Cell Oncol 31: 487-500, 2009.