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Aims: Tumour suppressor gene silencing through promoter hypermethylation plays an important role in oncogenesis. Carcinogenesis of hereditary cancers usually differs from that of their sporadic counterparts, but methylation has hardly been studied in hereditary ovarian cancer. The aim of this study was to investigate promoter methylation of a set of common tumour suppressor genes in *BRCA1*-related ovarian cancer in comparison with sporadic ovarian cancer.

Methods and results: Methylation-specific multiplex ligation-dependent probe amplification was used to assess the extent of promoter methylation of 24 different tumour suppressor genes in *BRCA1*-associated (n = 25) and matched sporadic ovarian tumours (n = 50). A cumulative methylation index (CMI) was calculated and differences between individual genes were analysed. There was no significant difference in cumulative methylation between *BRCA1*-associated and sporadic ovarian carcinomas (median CMI 108; CMI 110; P = 0.86). Also, methylation patterns of individual genes did not show distinct differences after correction for multiple comparisons. *CDH13*, *GSTP1* and *RASSF1* were frequently methylated in both sporadic and hereditary ovarian cancers. *BRCA1* methylation occurred in 14% of sporadic tumours, but was not detected in *BRCA1*-associated tumours.

Conclusions: *CDH13*, *GSTP1* and *RASSF1* are frequently methylated in both sporadic and *BRCA1*-associated ovarian cancers. Interestingly, methylation of *BRCA1*, while frequent in sporadic ovarian cancer, never occurred in the hereditary group. *BRCA1*-associated ovarian cancers mimic their sporadic counterparts in extent and pattern of promoter methylation of several common tumour suppressor genes. This finding could have implications for future chemotherapy regimens based on epigenetic changes.

Keywords: *BRCA1* gene, hereditary, methylation, MS-MLPA, neoplastic syndromes, ovarian neoplasms

Abbreviations: CMI, cumulative methylation index; MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification; PCR, polymerase chain reaction; UMCG, University Medical Centre Groningen; UMCU, University Medical Centre Utrecht

Introduction

Ovarian carcinoma is the fifth most lethal cancer for women in the Western world, accounting for more mortality than all other gynaecological malignancies combined. Two-thirds of patients present with advanced-stage disease.[1,2] One of the most important risk factors for ovarian carcinoma is a hereditary predisposition. About 8% of all ovarian carcinomas are associated with a *BRCA1* germ-line mutation;[3–5] these patients have a 40% lifetime risk of developing ovarian cancer, compared with 1% in the non-predisposed population.[6,7]

Breast and ovarian cancer development in hereditary predisposed women is primarily driven by germ-line mutations of the *BRCA1* or *BRCA2* genes. These genes play an important role in homologous recombination,

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a process that repairs double-strand DNA breaks. Defects in these mechanisms theoretically lead to an alternative carcinogenic pathway through which hereditary ovarian cancers arise. This has extensively been proven for *BRCA1*- and *BRCA2*-related breast cancers, which are known for their clearly different genetic makeup and associated immunophenotype and morphology.[8–13] Genetic differences have also been shown for *BRCA1/2* germ-line mutation-related ovarian cancer.[14–19]

Not only genetics but also epigenetic phenomena play a prominent role in oncogenesis. The best studied epigenetic modification is DNA methylation of cytosine residues by DNA methyltransferases. CpG islands within promoter regions are often unmethylated in normal cells as opposed to cytosines in the rest of the genome.[20,21] However, cancer cells typically acquire methylated CpG islands through the binding of methyl groups to cytosine dinucleotides.[20,22] Hypermethylation of CpG islands within the promoter region of a tumour suppressor gene (further denoted 'methylation') is associated with loss of gene function and thus results in a first and/or second hit in the Knudson's two-hit theory.[20,23] Methylation has hardly been studied in hereditary ovarian cancer. Since in a previous study less methylation in *BRCA1*-associated breast cancer compared with sporadic controls was found,[24] we set out to compare methylation of *BRCA1* germ-line mutation-related ovarian cancers with sporadic controls.

Materials and methods

PATIENTS

We compared the methylation pattern of 25 paraffin-embedded *BRCA1*-associated ovarian carcinomas with that of 50 sporadic carcinomas. Specimens collected between 1980 and 2007 were obtained from the archives of the Pathology Department of the University Medical Centre Utrecht (UMCU) and the University Medical Centre Groningen (UMCG) (the Netherlands). Use of anonymous or coded left-over material for scientific purposes is part of the standard treatment contract with patients in the UMCU and complies with the Dutch code of conduct for use of left-over tissue in the UMCG.[25] *BRCA1* germ-line mutational status had previously been confirmed in all *BRCA1*-associated cases by mutational analysis. Sporadic cases were unselected for family history.

Haematoxylin and eosin-stained slides were reviewed by a single pathologist (P.J.v.D.) to confirm diagnosis and select representative cancer cell-rich areas. Histological classification and staging were assessed according to the World Health Organization and International Federation of Gynecologists and Obstetricians systems[26] and grading was performed according to the percentage of solid areas and degree of nuclear pleomorphism.

All available hereditary cancers were matched to two sporadic cases based on age, grade and histological type. Table 1 shows the baseline clinicopathological characteristics.

DNA ISOLATION

Unstained slides (5 μm) were deparaffinized by standard methods. Roughly 100–200 mm^2 tumour tissue, bearing >80% cancer cells, was scraped off and suspended in 100 μl lysis buffer (50 mM Tris–HCl pH 8.0; 0.5% Tween 20). Proteinase K (100 μg) (Sigma, St Louis, MO, USA) was added, after which the sample was incubated overnight at 52°C. Next, heat inactivation was applied for 5 min at 99°C and samples were then immediately placed on ice. To spin down cell debris, samples were subsequently centrifuged for 30 min, 18 000 *g* at 4°C, after which the supernatant was stored at 4°C.

Table 1. Baseline characteristics of *BRCA1*-related and sporadic ovarian cancers

	BRCA1 (*n* = 25)	Sporadic (*n* = 50)	Total (*n* = 75)
Mean age (range)	50 (38–73)	55 (37–73)	54 (37–73)
Histological type (%)			
Serous	22 (88)	44 (88)	66
Endometrioid	3 (12)	6 (12)	9
Grade (%)			
1	1 (4)	2 (4)	3
2	6 (24)	12 (24)	18
3	18 (72)	36 (72)	54
FIGO stage (%)			
I	0 (0)	2 (4)	2
II	4 (16)	4 (8)	8
III	9 (36)	32 (64)	41
IV	1 (4)	6 (12)	7
Previous chemotherapy (%)			
Yes	2 (8)	10 (20)	12
No	12 (48)	34 (68)	46

METHYLATION-SPECIFIC MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION

In methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), ligation of probe oligonucleotides is followed by digestion of the DNA–probe complex with methylation-sensitive endonucleases, allowing quantification of methylation in archival patient material.[27] The MS-MLPA procedure has previously been described and validated.[27,28] MS-MLPA was performed using the ME-001B kit (MRC-Holland BV, Amsterdam, the Netherlands). In brief, approximately 100 ng isolated DNA in 5 μl TE (10 mM Tris, 1 mM ethylenediaminetetraacetic acid pH 7.5) was heat-denatured (10 min at 98°C) and incubated with a probe set containing probes with affinity to the promoters of 24 different tumour suppressor genes (Table 2) at 60°C for 16 h. After adding ligase buffer, samples were divided into two series of tubes and heated to 49°C. To the first series, ligase was added. To the second series, the same ligase and 5 U restriction enzyme *HhaI* (Promega Corp., Madison, WI, USA) was added. Ligation (both series) and digestion (second series) reactions were performed by incubating at 49°C for 30 min followed by inactivation at 98°C for 5 min. Polymerase chain reaction (PCR) buffer, PCR primers, enzyme dilution buffer, polymerase and 5 μl of each ligation or digestion sample were mixed and amplified by 35 PCR cycles (95°C for 30 s, 60°C for 30 s and 72°C for 1 min, finished by 20 min at 72°C). Fragment analyses were run on an ABI 310 sequencer (Applied Biosystems, Foster City, CA, USA). *SssI*-treated human sperm DNA was used as a positive control and human sperm DNA as a negative control.

Data were first normalized by dividing the peak area of single probe by a cumulative peak area of 15 control probes, not degraded by *HhaI*. Then, to calculate the percentage of methylated promoters of all promoter regions detected, normalized peaks from the digestion reaction were divided by normalized peaks from the undigested control reaction.

STATISTICS

A cumulative methylation index (CMI) was calculated as before[24] by the sum of the methylation percentages of all 24 genes shown in Table 2. The Mann–Whitney test was used to compare CMI and methylation of individual genes between *BRCA1*-associated and sporadic cancers and to compare CMI for different tumour types. The Kruskal–Wallis test was used to compare CMI between different histological grades and clinical stages. Spearman correlation coefficient was calculated for the association of CMI with age.

In a different approach, the number of cases with >15% methylation was registered. This cut-off value was previously determined based on titration

Table 2. Genes in the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) KIT ME001B Tumor suppressor-1 (MRC Holland)

Gene	Name	Gene	Name
TIMP3	Tissue inhibitor of metalloproteinase 3	*PTEN*	Phosphatase and tensin homologue
APC	Adenomatous polyposis coli	*BRCA2*	Breast cancer 2, early onset
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p14-ARF)	*CD44*	CD44 molecule (Indian blood group)
MLH1	MutL protein homologue 1	*RASSF1*	Ras association domain family member 1
ATM	Ataxia telangiectasia mutated	*DAPK1*	Death-associated protein kinase 1
RARB	Retinoic acid receptor, beta	*VHL*	von Hippel–Lindau
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15)	*ESR1*	Oestrogen receptor 1
HIC1	Hypermethylated in cancer 1	*TP73*	Tumour protein p73
CHFR	Checkpoint with forkhead and ring finger domains	*FHIT*	Fragile histidine triad gene
BRCA1	Breast cancer 1, early onset	*IGSF4*	Cell adhesion molecule 1 (CADM1)
CASP8	Caspase 8	*CDH13*	Cadherin 13, H-cadherin
CDKN1B	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	*GSTP1*	Glutathione S-transferase pi

experiments in cell lines and the effect of methylation on protein expression, and most accurately discriminated between tumour and normal tissue.[29] Chi-squared and Fisher's exact test were applied to compare frequency of methylation for individual genes of *BRCA1*-associated tumours with sporadic tumours.

Two-sided $P \leq 0.05$ was used as the threshold for significance. Correction for multiple comparisons was applied by resetting the 0.05 threshold to 0.05/24 = 0.002. Analysis was repeated, excluding patients receiving preoperative chemotherapy to evaluate the influence of this possible confounder. All statistical analyses were carried out with SPSS 15.0.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Most patients presented with an advanced stage of ovarian cancer (48/58) and the tumours were histologically classified as grade 3 (54/75) serous (66/75) carcinomas. Twelve out of 58 patients received preoperative chemotherapy.

When comparing sporadic and *BRCA1*-related cancers, there were no significant differences in quantitative methylation of individual genes after correction for multiple comparisons (Table 3). However, when considering the percentage of samples with >15% methylation (Table 4) it was interesting to note that none of the *BRCA1*-associated cancers showed methylation of the *BRCA1* gene exceeding 15%, while in the sporadic group seven (14%) cancers had a highly methylated *BRCA1* gene promoter (varying from 48% to 82%). For *ATM*, *BRCA2*, *CD44*, *CDKN1B*, *CHFR*, *DAPK1*, *ESR1*, *FHIT*, *HIC1*, *IGSF4*, *RARB* and *VHL* methylation did not exceed the 15% threshold, while the other genes did show relevant differences in methylation between sporadic and *BRCA1*-associated cancers (Table 4). There was no significant difference ($P = 0.86$) in median CMI between *BRCA1*-associated ovarian carcinomas (CMI = 108) and sporadic controls (CMI = 110).

There were no significant differences in cumulative methylation between ovarian tumours with different clinical stages ($P = 0.32$), histological subtypes ($P = 0.34$) or different grades ($P = 0.44$). Furthermore, no correlation was found ($r = -0.007$, $P = 0.95$) between age and cumulative methylation, nor between age and methylation of any gene in the study group.

Patients who received chemotherapy before surgery showed slightly less methylation (median CMI = 101) than those who did not (median CMI = 111), although this was not significant ($P = 0.16$). When excluding cases that received preoperative chemotherapy, there was also no significant difference ($P = 0.30$) in CMI between *BRCA1*-related ovarian carcinomas (median CMI = 108) and sporadic controls (median CMI = 117). When considering the percentage of samples with >15% methylation, the same trend for absence of *BRCA1* methylation in hereditary predisposed patients not receiving chemotherapy was seen.

In logistic regression, no combination of genes could be identified that could predict *BRCA1*-associated ovarian cancer.

Discussion

The purpose of this study was to investigate promoter methylation of a set of common tumour suppressor genes in *BRCA1* germ-line mutation-related and sporadic ovarian cancer. We applied a threshold of 15% methylation based on cell line experiments and methylation levels in normal tissue.[29] *ATM*, *BRCA2*, *CD44*, *CDKN1B*, *CHFR*, *DAPK1*, *ESR1*, *FHIT*, *HIC1*, *IGSF4*, *RARB* and *VHL* never showed methylation above this threshold, indicating that promoter methylation of these tumour suppressor genes may not play an important role in either hereditary or sporadic ovarian carcinogenesis. On the other hand, *APC*, *BRCA1*, *CASP8*, *CDH13*, *CDKN2A*, *CDKN2B*, *GSTP1*, *MLH1*, *PTEN*, *RASSF1*, *TIMP3* and *TP73* did show promoter methylation to a varying extent above the 15% threshold.

This is the first study using a quantitative method to determine methylation of tumour suppressor genes in ovarian cancer, which should be taken into account when comparing these results with previous non-quantitative studies. Various percentages of methylation have been reported for *GSTP1*, *CDH13*, *RASSF1*, *BRCA1*, *CDKN2A*, *APC*, *CASP8*, *TP73*, *CDKN2B*, *HIC1* and *RARB*.[30–35] For *CDH13*, *RASSF1*, *BRCA1*, *CDKN2A*, *CASP8*, *TP73* and *CDKN2B* our findings largely correspond to published data. Strikingly, we found that 48% percent of tumours showed *GSTP1* methylation, while in previous studies this was only 0–9%.[31–33] On the other hand, *APC* methylation in this study (6%) was much lower than reported (18–47%).[31,32,34,35] Moreover, we found no methylation of *HIC1* and *RARB*, compared with 15–52%[30,31,34] and 2–17%,[31,32,34] respectively, in the literature.

We did not find a significant difference in cumulative methylation or methylation of individual genes when comparing *BRCA1*-associated ovarian carcinomas with clinicopathologically matched sporadic carcinomas. This finding supports the notion that sporadic cancers can have a *BRCA1* phenotype[19] and could indicate that epigenetic changes play an equally important role

Table 3. Median promoter methylation percentage (range) by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) for 24 tumour suppressor genes in *BRCA1*-related and control sporadic ovarian cancers

	BRCA1		Sporadic		
Gene	Mean (%) (SD†)	Median (%) (range)	Mean (%) (SD†)	Median (%) (range)	*P*-value*
APC	4.7 (15.4)	0.0 (0–71)	3.1 (10.7)	0.0 (0–52)	0.50
ATM	4.1 (2.2)	3.7 (0–9)	2.6 (1.9)	2.8 (0–6)	0.01
BRCA1	2.3 (2.1)	2.1 (0–8)	9.9 (22.2)	1.8 (0–82)	0.56
BRCA2	3.3 (3.3)	3.6 (0–11)	2.4 (2.4)	2.6 (0–8)	0.33
CASP8	3.5 (9.0)	0.0 (0–45)	4.1 (12.6)	0.0 (0–79)	0.75
CD44	4.6 (3.4)	4.7 (0–12)	4.8 (2.8)	4.8 (0–12)	0.87
CDH13	16.0 (13.6)	11.3 (0–48)	21.5 (23.0)	13.5 (0–100)	0.45
CDKN1B	1.2 (2.0)	0.0 (0–8)	0.5 (1.2)	0.0 (0–5)	0.18
CDKN2A	7.7 (5.0)	7.7 (0–21)	5.4 (5.5)	5.7 (0–18)	0.05
CDKN2B	4.2 (3.4)	3.5 (0–11)	2.2 (3.0)	1.6 (0–17)	0.01
CHFR	0.0 (0.0)	0.0 (0–0)	0.0 (0.0)	0.0 (0–0)	1.00
DAPK1	0.6 (1.7)	0.0 (0–8)	0.6 (1.5)	0.0 (0–6)	0.98
ESR1	1.9 (3.3)	0.0 (0–11)	0.6 (1.7)	0.0 (0–6)	0.05
FHIT	0.4 (1.4)	0.0 (0–6)	0.1 (0.9)	0.0 (0–6)	0.55
GSTP1	17.7 (10.2)	15.2 (5–47)	14.9 (4.5)	14.8 (6–26)	0.55
HIC1	5.3 (4.1)	4.6 (0–13)	3.6 (2.8)	3.9 (0–12)	0.14
IGSF4	2.2 (2.9)	0.0 (0–10)	1.6 (2.4)	0.0 (0–9)	0.41
MLH1	3.9 (11.1)	1.4 (0–57)	1.0 (1.0)	1.0 (0–4)	0.06
PTEN	10.0 (4.8)	8.9 (0–20)	8.6 (3.2)	8.5 (0–15)	0.43
RARB	5.9 (2.7)	5.5 (0–12)	4.8 (2.7)	4.7 (0–13)	0.06
RASSF1	10.1 (16.7)	5.2 (0–79)	15.6 (24.7)	3.8 (0–93)	0.94
TIMP3	0.3 (1.0)	0.0 (0–4)	0.8 (4.5)	0.0 (0–32)	0.97
TP73	7.8 (5.7)	7.2 (0–23)	7.2 (4.3)	7.8 (0–17)	0.95
VHL	0.1 (0.6)	0.0 (0–3)	0.1 (0.6)	0.0 (0–4)	1.00
Cumulative	118 (55)	108 (35–236)	116 (54)	110 (9–248)	0.86

*Mann–Whitney.
†Standard deviation.

in hereditary and sporadic ovarian cancers. Interestingly, none of the hereditary tumours compared with 14% of the sporadic tumours showed >15% methylation of the *BRCA1* gene. This implies that a second hit due to methylation of the *BRCA1* gene does not occur in hereditary ovarian cancer, which is consistent with findings by others.[31,36] For clinical practice, demethylating the *BRCA1* gene in order to overcome chemotherapeutic resistance[37] will therefore not be useful in *BRCA1* germ-line mutation carriers.

Table 4. Number of samples with >15% methylation of specific genes per group

Gene	BRCA-1-associated tumours (Total *n* = 25) (%)	Sporadic tumours (Total *n* = 50) (%)	*P*-value*	Total (%)
APC	2 (8)	3 (6)	1.00*	5 (7)
BRCA1	0 (0)	7 (14)	0.09*	7 (9)
CASP8	1 (4)	3 (6)	1.00*	4 (5)
CDH13	8 (32)	22 (44)	0.32†	30 (40)
CDKN2A	3 (12)	3 (6)	0.39*	6 (8)
CDKN2B	0 (0)	1 (2)	1.00*	1 (1)
GSTP1	13 (52)	24 (48)	0.74†	37 (49)
MLH1	1 (4)	0 (0)	0.33*	1 (1)
PTEN	4 (16)	2 (4)	0.09*	6 (8)
RASSF1	4 (16)	12 (24)	0.43†	16 (21)
TIMP3	0 (0)	1 (2)	1.00*	1 (1)
TP73	3 (12)	1 (2)	0.11*	4 (5)

Only genes that showed >15% methylation in at least one tumour are shown.
*Fisher's exact test.
†χ^2 test.

These results are in contrast with the findings we have previously described in hereditary breast cancer, where we showed lower levels of methylation for 11 tumour suppressor genes in *BRCA1*-associated compared with sporadic breast cancer.[24] Conversely, Esteller *et al.*[38] found no difference in overall promoter methylation between hereditary and sporadic breast and colon cancer. Of note, genes studied in these studies only partly overlap with those presently studied in hereditary ovarian cancer.

We found no clear influence on methylation when tumours had been treated with chemotherapy before surgery, which is in line with previous findings by Swisher *et al.*[39] A correlation between age and methylation has been described before,[23,24,40,41] but was not found in ovarian cancer in the present study. In addition, we found no associations between methylation and stage, grade and type. With a mean age of 55 years and high-grade, mostly serous cancers, our sporadic study population resembles the known characteristics of *BRCA1*-associated ovarian cancers.[42,43] Although matching for these features appears to be the optimal study set-up, this approach has some limitations. Available evidence suggests that each of the histological subtypes of ovarian cancer is associated with distinct molecular alterations. High-grade serous and endometrioid carcinomas have been proposed to have early mutations in *TP53* and loss of *BRCA1* or *BRCA2*.[44,45] Our study population consisted of high-grade, serous or endometrioid tumours, which may share elements of their oncogenetic pathways and consequently have similar methylation patterns.

In conclusion, *CDH13*, *GSTP1* and *RASSF1* are frequently methylated in both sporadic and hereditary ovarian cancers. Interestingly, frequent methylation of *BRCA1* in sporadic ovarian cancer never occurred as a second hit in the hereditary group. Ovarian cancers in *BRCA1* germ-line mutation carriers mimic their sporadic counterparts in extent and pattern of promoter methylation of 24 common tumour suppressor genes.

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