Promoter hypermethylation patterns in fallopian tube epithelium of *BRCA1* and *BRCA2* germ line mutation carriers

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Abstract

BRCA1/2 germ line mutation carriers have a high risk of developing fallopian tube carcinoma (FTC), thought to occur through different early (p53 signatures) and later (dysplasia, intra-epithelial carcinoma) premalignant stages. Promoter hypermethylation of tumour suppressor genes is known to play a key role in (early) carcinogenesis. However, little is known about methylation in normal and (pre)malignant fallopian tube tissue. We identified 14 areas of p53 accumulation in the fallopian tubes of *BRCA* mutation carriers. Cells from these areas were harvested together with cells from adjacent benign appearing areas. An age-matched non-*BRCA* sporadic control group ($n=13$) and eight sporadic FTCs were included as negative and positive controls respectively. Methylation-specific multiplex ligation-dependent probe amplification was used to assess promoter methylation of 70 tumour suppressor genes in all samples. We observed a gradual increase in methylation from sporadic control tissue (median cumulative methylation index (CMI) 568.19) through normal tissue and from areas of p53 accumulation in *BRCA* carriers (median CMI 687.54 and 676.72) to FTC (median CMI 780.97). Furthermore, the methylation percentage of many individual tumour suppressor genes differed significantly between these groups, gradually increasing as for CMI. Between areas with and without p53 accumulation in *BRCA* mutation carriers no significant differences were found. In this paper, we have shown that *BRCA* mutation carriers display increased methylation of tumour suppressor genes in their non-malignant fallopian tube epithelium, closer to methylation levels in FTC than to normal sporadic tissue. Methylation could, therefore, play an important role in the increased risk of gynaecological malignancies in *BRCA* mutation carriers.

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Introduction

BRCA mutation carriers are at hereditary high risk of developing serous ovarian cancer. For *BRCA1* mutation carriers, the average cumulative risk to develop ovarian cancer by age 70 is 39% (confidence interval (CI): 18–54%) and for *BRCA2* carriers 11% (CI: 2.4–19%; Antoniou *et al*. 2003). Apart from ovarian cancer, these women are also susceptible to develop other gynaecological malignancies such as serous fallopian tube carcinoma (FTC; Zweemer *et al*. 2000, Cass *et al*. 2005, Callahan *et al*. 2007) and serous peritoneal carcinoma (Menczer *et al*. 2003, Finch *et al*. 2006).

Furthermore, dysplasia (including tubal intra-epithelial carcinoma) arising from the fallopian tubes, which is an established precursor of FTC and probably also serous ovarian carcinoma, is also more common in *BRCA* mutation carriers (Piek *et al*. 2001, Hermsen *et al*. 2006).

These dysplastic lesions share a high rate of *TP53* mutations with serous invasive carcinoma and often (79–100%) show p53 accumulation by immunohistochemistry (Lee *et al*. 2007*b*, Shaw *et al*. 2009; Fig. 1).

Recently, another lesion arising in the fallopian tube, the p53 signature, has been hypothesised to be an even

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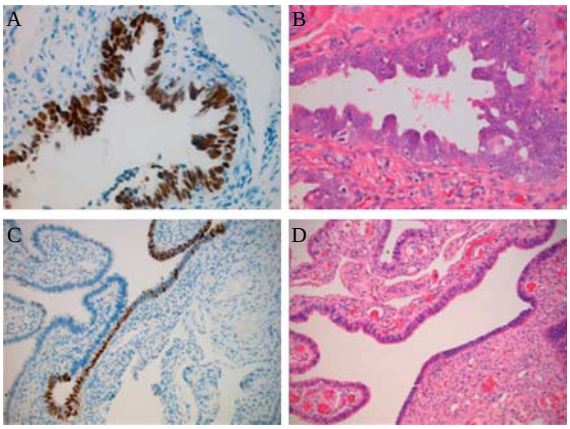

Figure 1 (A) p53 accumulation in human fallopian tube tissue in combination with dysplasia (p53 staining with anti-p53 mouse antibody); (B) corresponding haematoxylin–eosin staining (A and B at 400× magnification). (C) p53 accumulation in morphologically benign human fallopian tube tissue (p53 signature); (D) corresponding haematoxylin–eosin staining (C and D at 200× magnification).

earlier stage in the pathway leading to the development of serous gynaecological malignancies (Crum 2009).

p53 signatures are defined as having strong nuclear staining for p53 in morphologically benign appearing tissue for at least 12 consecutive cells (Fig. 1). They have been reported to occur more frequently in the fimbriae than in the proximal fallopian tubes, thereby coinciding with the region in which both dysplastic lesions and early invasive serous carcinomas in *BRCA* mutation carriers are most often observed (Lee *et al.* 2007*b*). Like dysplastic lesions, p53 signatures were not found in ovarian surface epithelium and cortical inclusion cysts (Piek *et al.* 2003, Folkins *et al.* 2008).

Monitoring epigenetic changes is a relatively novel way of tracking oncogenetic progression. Of these changes, promoter hypermethylation is particularly useful since it occurs early during carcinogenesis and could be utilised to more fully comprehend the *BRCA* mutation-driven carcinogenetic pathway (Suijkerbuijk *et al.* 2008).

Methylation can occur at sites where a cytosine nucleotide is followed by guanine (CpG dinucleotides). In many genes, groups of these dinucleotides (CpG islands) are present in the promoter regions (Ng & Bird 1999). Hypermethylation of these CpG islands usually shuts down transcription of these genes and is thereby a common gene inactivation mechanism. In physiological conditions, this occurs in, e.g. silenced alleles on the X-chromosome of females (Surani 1998). In cancer, methylation is frequently observed in promoter regions of tumour suppressor genes, leading to their inactivation (Esteller 2008). Promoter hypermethylation (further denotes 'methylation') thereby seems to play a role in carcinogenesis.

Indeed, methylation is seen in premalignant lesions and even in normal tissue adjacent to cancer (Yan *et al.* 2006, Tam *et al.* 2007, Suijkerbuijk *et al.* 2008, García *et al.* 2009, Park *et al.* 2009).

For ovarian cancer, methylation has been shown in a number of tumour suppressor genes such as *RASSF1, APC, PTEN, BRCA1, RARβ, CDH13* and *HIC1* (Rathi *et al.* 2002, Makarla *et al.* 2005, Choi *et al.* 2006, Tam *et al.* 2007). For FTC and its precursors, methylation has not yet been described.

Our aim was, therefore, to study methylation in normal, premalignant and malignant fallopian tube tissue from sporadic patients and *BRCA* mutation carriers to gain insight in the role of methylation in sporadic and *BRCA* mutation-driven fallopian tube carcinogenesis.

Materials and methods

Patient selection

We selected women documented to have either a *BRCA1* or a *BRCA2* mutation, who consequently underwent a prophylactic bilateral adenectomy at the University Medical Centre Utrecht between January 1996 and November 2009.

An age-matched control (sporadic) group consisted of patients not tested for a *BRCA* mutation because of unremarkable family history, who underwent salpingectomy for reasons other than suspected gynaecological malignancies. Exclusion criteria were history of breast cancer and histological abnormalities including inflammation and the presence of dysplastic lesions. A third group of women with FTC not tested for a *BRCA* mutation was included, who underwent surgery between 1993 and January 2010. Finally, ovaries from four *BRCA* and four non-*BRCA* control patients collected at Brigham and Women's Hospital, Boston, MA, USA, were included for analysis.

Immunohistochemistry

Fallopian tube tissue obtained after surgery was formalin fixed and paraffin embedded. From each patient, the fimbriae were identified by review of haematoxylin- and eosin-stained slides by two experienced pathologists (PvD/DG) and screened for dysplastic areas (including intra-epithelial carcinoma) according to established definitions (Piek *et al.* 2001, 2003). Slides containing the largest amount of fimbriae and/or the largest amount of fallopian tube tissue were selected. Subsequently, 4 μm sections of

paraffin-embedded tissue were mounted on pre-coated slides. The tissue was dewaxed with xylene and rehydrated through graded alcohol according to standard procedures.

After antigen retrieval in citrate buffer for 20 min at 100 °C, endogenous peroxidase activity was blocked with 1.5% H_2O_2 for 5 min and slides were incubated at room temperature for 15 min with BP53-12-1 anti-p53 monoclonal mouse antibody (1:100 in PBS-BSA; BioGenex Labs, San Ramon, CA, USA). Staining was developed with the Novolink polymer (Vision Biosystems, Newcastle, UK) for 8 min.

Counterstaining was done with haematoxylin. Appropriate positive and negative controls were used. All staining was done using the Bond-max stainer (Leica, Wetzlar, Germany).

p53 was considered accumulated by immunohistochemistry if nuclear detail was obscured for at least 12 adjacent nuclei (Lee *et al*. 2007*b*). Images were taken using a Leica digital camera DMX1200 through a 20× and 40× objective.

DNA isolation

Epithelial cells from the fallopian tubes and ovarian cortex were harvested from 4 μm sections of paraffin-embedded tissue using micro-dissection. DNA isolation was achieved by suspending in direct lysis buffer (50 mM Tris–HCl, pH 8.0, 0.5% Tween 20), after which proteinase K was added for 60 min at 56 °C. Proteinase K was then inactivated by incubation at 100 °C for 10 min and the supernatant was used for further analysis. DNA content was measured using a spectrophotometer.

Methylation analysis

Methylation status was assessed using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA; MRC Holland, Amsterdam, The Netherlands) for a set of 70 tumour suppressor genes (probe mixes ME001-C1, ME002-B1, ME003-A1 and ME004-A1; MRC Holland) as described previously by Nygren *et al*. (2005), Bol *et al*. (2010) and Seeber *et al*. (2010) and which was partly developed specifically for adnexal tumours (Nowee *et al*. 2007; Table 1).

The isolated DNA was denatured by heating for 10 min at 98 °C. The probe mixes were added and the sample was incubated overnight at 60 °C. Each sample was divided into half; to the first half, ligase buffer was added and to the second half, both ligase buffer and a methylation-sensitive restriction enzyme (Hha1) were added. Next, PCR buffer and primers were added and the sample was run for 35 repeated cycles in PCR.

All cases were run in duplicate, using the mean methylation percentage of the two runs in subsequent analyses.

Reaction products were separated by capillary electrophoresis (on an ABI 310 automated DNA sequencer; Applied Biosystems, Foster City, CA, USA). Analysis was performed using GeneMapper Software (Applied Biosystems) and subsequently Coffalyser Software (MRC Holland). Based on previous cell line experiments (Gylling *et al*. 2007, Joensuu *et al*. 2008), we considered a promoter to show methylation if the methylation dosage ratio was ≥ 0.15, which corresponds to 15% of DNA methylated.

The cumulative methylation index (CMI) was calculated as the sum of the percentages of methylation of the individual genes as before (Suijkerbuijk *et al*. 2008, Seeber *et al*. 2010). The CMI was also calculated for only genes showing hypermethylation ($\geq 15\%$) and frequently methylated genes (in $\geq 20\%$ of cases).

Statistical analysis

Mann–Whitney and Kruskal–Wallis tests were used to compare methylation between normal sporadic tissue, normal tissue of *BRCA* mutation carriers, areas with p53 accumulation of *BRCA* mutation carriers and FTC. Wilcoxon sign rank test was performed to compare methylation between normal tissue of *BRCA* mutation carriers and age-matched sporadic controls, and between normal tissue of *BRCA* mutation carriers and areas of p53 accumulation in *BRCA* mutation carriers. All statistical analyses were performed using SPSS 16.0 for Windows (IBM Corporation, Armonk, NY, USA).

Results

Patient characterisation

The original group of *BRCA* mutation carriers consisted of 88 women, of whom 62 had a *BRCA1* mutation, 25 a *BRCA2* mutation and one had both. From these women, bilateral fallopian tubes were examined, except for three cases in which only a unilateral tube was removed during surgery. Fourteen cases (mean age 46.5 years; nine *BRCA1* mutation carriers and five *BRCA2* mutation carriers) showed one or more areas with p53 accumulation, resulting in their inclusion for promoter methylation status analysis. The age-matched control (sporadic) group consisted of 13 patients (mean age 49.5 years) and the FTC group with eight patients (mean age 67 years). The groups for

Table 1 List of tumour suppressor genes studied for methylation in human fallopian tube tissue

Gene symbol	Gene name
TP73	Tumour protein p73
CASP8	Caspase 8, apoptosis-related cysteine peptidase
VHL	von Hippel–Lindau tumour suppressor
RARβ	Retinoic acid receptor, beta
MLH1	mutL (*Escherichia coli* (*E. coli*)) homologue 1 (colon cancer, non-polyposis type 2)
RASSF1	Ras association (RalGDS/AF-6) domain family member 1
FHIT	Fragile histidine triad gene
APC	Adenomatous polyposis coli
ESR1	Oestrogen receptor 1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2B	Cyclin-dependent kinase inhibitor 2B
DAPK1	Death-associated protein kinase 1
PTEN	Phosphatase and tensin homologue
CD44	CD44 molecule (Indian blood group)
GSTP1	Glutathione *S*-transferase pi 1
ATM	Ataxia telangiectasia mutated
IGSF4	Immunoglobulin superfamily 4
CDKN1B	Cyclin-dependent kinase inhibitor 1B
CHFR	Checkpoint with forkhead and ring finger domains
BRCA2	Breast cancer 2, early onset
CDH13	Cadherin 13, H-cadherin
HIC1	Hypermethylated in cancer 1
BRCA1	Breast cancer 1, early onset
TIMP3	Tissue inhibitor of metallopeptidase 3
MSH6	mutS homologue 6 (*E. coli*)
PAX5	Paired box 5
MGMT	*O*-6-methylguanine-DNA methyltransferase
PAX6	Paired box 6
WT1	Wilms tumour 1
CADM1	Cell adhesion molecule 1
RB1	Retinoblastoma 1
THBS1	Thrombospondin 1
PYCARD	PYD and CARD domain containing
TP53	Tumour protein p53
STK11	Serine/threonine kinase 11
GATA5	GATA-binding protein 5
PRDM2	PR domain containing 2
RUNX3	Runt-related transcription factor 3
HLTF	Helicase-like transcription factor
SCGB3A1	Secretoglobin, family 3A, member 1
ID4	Inhibitor of DNA binding 4
TWIST1	Twist homologue 1 (*Drosophila*)
SFRP4	Secreted frizzled-related protein 4
DLC1	Deleted in liver cancer 1
SFRP5	Secreted frizzled-related protein 5
H2AFX	H2A histone family, member X
CCND2	Cyclin D2
CACNA1G	Calcium channel, voltage-dependent, T-type, alpha 1G subunit
TGIF	TGFB-induced factor homeobox
BCL2	B-cell CLL/lymphoma 2
CACNA1A	Calcium channel, voltage-dependent, P/Q-type, alpha 1A subunit
EPHB2	EPH receptor B2
LMNA	Lamin A/C
NTRK1	Neurotrophic tyrosine kinase, receptor, type 1
RARRES1	Retinoic acid receptor responder (tazarotene induced) 1
TERT	Telomerase reverse transcriptase
IGF2R	Insulin-like growth factor 2 receptor
GATA4	GATA-binding protein 4
SFRP1	Secreted frizzled-related protein 1
MEN1	Multiple endocrine neoplasia I
MUS81	MUS81 endonuclease homologue (*Saccharomyces cerevisiae*)
RBM14	RNA-binding motif protein 14
WIF1	WNT inhibitory factor 1
APAF1	Apoptotic peptidase activating factor 1
DNAJC15	DnaJ (Hsp40) homologue, subfamily C, member 15
PCCA	Propionyl coenzyme A carboxylase, alpha polypeptide
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)
NF1	Neurofibromin 1
PCNA	Proliferating cell nuclear antigen
PXMP4	Peroxisomal membrane protein 4

analysis of the ovarian cortex consisted of four *BRCA* and four non-*BRCA* control patients (mean age 54.75 and 51.25 years respectively).

Frequently methylated genes

In normal sporadic tissue, 14 genes were frequently methylated, whereas 20 genes showed frequent methylation in normal tissue of *BRCA* mutation carriers and 19 in areas with p53 accumulation. In FTC, there were 20 frequently methylated genes (Table 2).

Promoter methylation in normal sporadic vs BRCA tissue

Normal *BRCA* tissue showed a significantly higher median methylation percentage than normal sporadic tissue for 19 genes (*CASP8, VHL, ATM* (2×, with different probes), *TIMP3, TP73, MSH6, PAX5, PTEN, MGMT, BRCA2, RB1, THBS1, CDH13, SFRP4, DLC1, APAF1, DNAJC15, PCNA* and *PXMP4*). Only for *WIF1*, median methylation was lower in normal *BRCA* tissue (Table 3). The average number of methylated genes was 8.54 (range 3–15) in normal sporadic tissue vs 11.92 (range 4–21) in normal *BRCA* tissue ($P=0.084$) and vs 12.71 (range 7–21) in p53-accumulated *BRCA* tissue ($P=0.038$).

Table 2 Genes showing frequent (≥20% of cases) promoter methylation in different types of human fallopian tube tissue (indicated by +)

	Normal sporadic fallopian tube tissue	Normal *BRCA* fallopian tube tissue	Areas of p53 accumulation in *BRCA* carriers	Sporadic fallopian tube carcinoma
RASFF1				+
APC				+
GSTP1				+
CDH13	+	+	+	+
TP73		+		
MSH6	+	+	+	+
MGMT		+		
CD44			+	
BRCA2		+	+	+
RB1	+	+		
TP53	+			
STK11	+			
RUNX3	+	+	+	+
H2AFX				+
CCND2			+	+
NTRK1		+	+	
TERT	+	+	+	+
IGF2R		+	+	+
TWIST1	+	+	+	+
GATA4	+	+	+	+
SFRP1		+	+	+
PTEN	+	+	+	+
PAX6	+	+	+	
MUS81		+	+	+
APAF1	+	+	+	+
DNAJC15	+	+	+	+
PCCA	+	+	+	+
NF1		+	+	+

Promoter methylation in normal sporadic tissue vs sporadic FTC

When comparing normal sporadic tissue with FTC, methylation of 35 genes differed significantly. For 30 of these, the methylation percentage was higher in FTC tissue (*CASP8, RARB, APC, CD44, IGSF4, CDKN1B, MSH6, GSTP1, ATM, CHFR, BRCA2, THBS1, HLTF, SCGB3A1, SFRP4, DLC1* (2×, with different probes), *SFRP5* (2×, with different probes), *H2AFX, CACNA1G, BCL2, CACNA1A, IGF2R, MEN1, RBM14, APAF1* (2×, with different probes), *DNAJC15, CDH1, NF1, PCNA* and *PXMP4*). *RB1, STK11, RUNX3, PAX6* and *WIF1* showed a lower methylation percentage in FTC (Table 3).

The average number of methylated genes in normal sporadic tissue was 8.54 (range 3–15) vs 12.88 (range 7–23) for FTC ($P=0.094$).

Promoter methylation in normal BRCA tissue vs p53-accumulated BRCA tissue

MGMT and *RB1* showed a significantly higher methylation percentage in normal *BRCA* tissue compared with *BRCA* areas with p53 accumulation (Table 3).

The average number of methylated genes was 11.92 (range 4–21) in areas without p53 accumulation and 12.71 (range 7–21) in areas with p53 accumulation (not significant).

Between the 14 paired areas with and without p53 accumulation, three cases showed significantly more methylation in areas with p53 accumulation and five in areas without accumulation.

Promoter methylation in BRCA tissue vs sporadic FTC

Between p53-accumulated areas in *BRCA* mutation carriers and FTC, a difference was found for seven genes. Five of these showed increased methylation in FTC (*CASP8, SFRP5* (2×, with different probes), *MEN1, RBM14* and *PXMP4*); the other two (*PAX5* and *PAX6*) showed the opposite trend (Table 3).

In areas without p53 accumulation in *BRCA* mutation carriers, a difference was found for 11 genes, of which seven showed increased methylation in FTC (*CASP8, GSTP1, PYCARD, CACNA1G, SFRP5* (2×, with different probes), *MUS81* and

Table 3 Comparison of methylation percentages of tumour suppressor genes between specified groups in human fallopian tube tissue using Mann–Whitney statistical analysis

	Group a vs Group b		Group a vs Group c		Group a vs Group d		Group b vs Group c		Group b vs Group d		Group c vs Group d	
Gene/probe	**Mean rank**	***P* value**	**Mean rank**	***P* value**	**Mean rank**	***P* value**	**Mean rank**	***P* value**	**Mean rank**	***P* value**	**Mean rank**	***P* value**
CASP8_2761	10.42 vs 17.32	0.016	10.77 vs 17.00	0.028	7.31 vs 17.00	0.000			8.86 vs 16.12	0.011	8.29 vs 17.12	0.002
VHL_3810	10.88 vs 16.89	0.034										
RARβ_4040					8.65 vs 14.81	0.024						
APC_1905					8.65 vs 14.81	0.023						
CD44_3817					8.35 vs 15.31	0.010						
GSTP1_1638									9.25 vs 15.44	0.030		
ATM_4044	10.77 vs 17.00	0.033	10.15 vs 17.57	0.012								
IGSF4_3819					8.46 vs 15.12	0.011						
CDKN1B_7949					8.50 vs 15.06	0.014						
TIMP3_2255	10.85 vs 16.93	0.015	10.62 vs 17.14	0.023								
TP73_1684	10.85 vs 16.93	0.046	10.65 vs 17.11	0.034								
MSH6_1250	9.42 vs 18.25	0.004	10.50 vs 17.25	0.027	7.77 vs 16.25	0.002						
VHL_3818			17.62 vs 10.64	0.022								
CDKN2A_1530									13.61 vs 7.81	0.041		
PAX5_3750	9.04 vs 18.61	0.002	10.73 vs 17.04	0.039					14.43 vs 6.38	0.005	13.64 vs 7.75	0.040
MGMT_5670							17.82 vs 11.18	0.032				
GSTP1_2747					9.00 vs 14.25	0.049						
ATM_3023	9.96 vs 17.75	0.0009	10.88 vs 16.89	0.043	8.58 vs 14.94	0.019						
CHFR_2737					8.81 vs 14.56	0.027						
BRCA2_2285	10.38 vs 17.36	0.022			7.92 vs 16.00	0.004						
RB1_2734	9.46 vs 17.54	0.006					16.65 vs 10.35	0.035				
RB1_4502					14.19 vs 5.81	0.003						
THBS1_1678	8.73 vs 18.89	0.001			9.15 vs 14.00	0.048						
PYCARD_2252									9.64 vs 14.75	0.016		
CDH13_2257	10.77 vs 17.00	0.041										
STK11_6783					12.38 vs 8.75	0.044						
RUNX3_11131					13.08 vs 7.62	0.050			13.61 vs 7.81	0.044		
HLTF_2758			10.96 vs 16.82	0.038	7.77 vs 16.25	0.001						
SCGB3A1_3305					8.73 vs 14.69	0.029						
SFRP4_3744	11.15 vs 16.64	0.038			8.58 vs 14.94	0.009						
DLC1_2754			10.23 vs 17.50	0.013	7.65 vs 16.44	0.001						
DLC1_2753	10.65 vs 17.11	0.017			8.73 vs 14.69	0.004						
SFRP5_9149					7.65 vs 16.44	0.001			8.43 vs 16.88	0.006	8.71 vs 16.38	0.006
SFRP5_9148					7.77 vs 16.25	0.001			8.32 vs 17.06	0.002	8.25 vs 17.19	0.001
H2AFX_8509					8.27 vs 15.44	0.007						
CACNA1G_10123					8.54 vs 15.00	0.020			9.36 vs 15.25	0.002		
BCL2_10352					8.69 vs 14.75	0.021						
CACNA1A_09055					8.96 vs 14.31	0.049						
IGF2R_2801					8.92 vs 14.38	0.045						
SFRP1_10564			10.19 vs 17.54	0.016								
PAX6_3748					13.96 vs 6.19	0.005			14.15 vs 5.88	0.003	15.00 vs 5.38	0.001
MUS81_10574									8.38 vs 15.25	0.013		
MEN1_9167					8.19 vs 15.56	0.006			8.42 vs 15.19	0.013	8.61 vs 16.56	0.004
RBM14_9429					8.38 vs 15.25	0.012					8.50 vs 16.75	0.004

Table 3 *continued*

Gene/probe	Group a vs Group b		Group a vs Group c		Group a vs Group d		Group b vs Group c		Group b vs Group d		Group c vs Group d	
	Mean rank	*P* value	Mean rank	*P* value	Mean rank	*P* value	Mean rank	*P* value	Mean rank	*P* value	Mean rank	*P* value
WIF1_10361	16.65 vs 10.35	0.035			13.38 vs 7.12	0.025						
APAF1_9166	10.31 vs 16.69	0.032			8.04 vs 15.81	0.005						
APAF1_9165					8.38 vs 15.25	0.014						
DNAJC15_3297	10.42 vs 16.58	0.040			8.00 vs 15.88	0.005						
CDH1_10364					8.62 vs 14.88	0.016						
NF1_3845					8.46 vs 15.12	0.011						
PCNA_3955	10.50 vs 16.50	0.038			8.81 vs 14.56	0.033						
PXMP4_3304	11.00 vs 16.00	0.015			8.00 vs 15.88	0.000					9.14 vs 15.62	0.008

Group a, normal sporadic tissue; Group b, BRCA tissue without p53 accumulation; Group c, BRCA tissue with p53 accumulation; Group d, fallopian tube carcinoma.

MEN1); the opposite was seen in four genes (*CDKN2A, PAX5, RUNX3* and *PAX6*; Table 3).

CMI between groups

A trend for an increasing CMI was observed from normal sporadic tissue (median CMI 568.19) through tissue of *BRCA* mutation carriers (median CMI 676.72 in areas with p53 accumulation and 687.54 in areas without p53 accumulation) to FTC (median CMI 780.97; $P=0.177$).

The median CMI of all genes was significantly higher in FTC than in normal sporadic tissue ($P=0.036$). Comparison of the other groups did not reach significance (normal sporadic vs normal *BRCA* tissue, $P=0.118$; p53-accumulated *BRCA* tissue vs FTC, $P=0.306$; and *BRCA* tissue with vs without p53 accumulation, $P=0.808$; Table 4).

Comparison of the CMI of only those genes showing hypermethylation ($\geq 15\%$) again showed a significant difference between normal sporadic tissue and FTC ($P=0.025$) and borderline significance when comparing normal sporadic tissue with normal *BRCA* tissue ($P=0.054$).

Kruskal–Wallis analysis of all groups provided a *P* value of 0.082 (Table 4).

When comparing the median CMI of the genes frequently hypermethylated in all groups, comparison of normal sporadic with normal *BRCA* tissue as well as normal sporadic tissue with FTC showed a significant difference ($P=0.018$ and $P=0.007$ respectively). Comparison of tissue of *BRCA* mutation carriers and FTC and of areas with and without p53 accumulation in *BRCA* mutation carriers did not reach significance ($P=0.183$ and $P=0.645$ respectively).

Kruskal–Wallis analysis of all groups showed significant population differences ($P=0.027$; Table 4).

Promoter methylation in normal cortex from BRCA and non-BRCA ovaries

The median CMI of all genes was higher in the cortex of *BRCA* ovaries (1636.83) than in non-*BRCA* ovaries (1220.25; $P=0.724$), as was the CMI of only the genes showing hypermethylation ($\geq 15\%$; 1242.17 vs 826.25) ($P=0.495$). The average number of methylated genes was 37.67 in *BRCA* ovaries and 27.75 in non-*BRCA* ovaries ($P=0.724$).

Twenty-nine genes were found to be frequently methylated in normal ovarian tissue, *ESR1, DAPK1, TIMP3*, ***MSH6***, *PAX5, CD44*, ***CDH13***, *PRDM2*, ***RUNX3***, *SCGB3A1, SFRP4, H2AFX, CCND2, CACNA1G, TIMP3, EPHB2*, ***TERT, IGF2R, TWIST1, GATA4,***

Table 4 Comparison of cumulative methylation index between different groups of human fallopian tube tissues using Mann–Whitney and Kruskal–Wallis statistical analyses

	CMI_{all}		CMI_{hyper}		CMI_{freq}	
	***P* value**	**Mean rank**	***P* value**	**Mean rank**	***P* value**	**Mean rank**
Normal sporadic vs BRCA tissue without p53 accumulation	0.118	11.15 vs 15.85	0.054	10.62 vs 16.38	0.018	9.96 vs 17.04
Normal sporadic vs BRCA tissue with p53 accumulation	0.308	12.38 vs 15.50	0.109	11.46 vs 16.36	0.099	11.38 vs 16.43
Normal sporadic tissue vs FTC	0.036	8.77 vs 14.62	0.025	8.62 vs 14.88	0.007	8.15 vs 15.62
BRCA tissue without vs BRCA tissue with p53 accumulation	0.808	14.38 vs 13.64	0.884	14.23 vs 13.79	0.645	14.73 vs 13.32
BRCA tissue with p53 accumulation vs FTC	0.306	10.43 vs 13.38	0.275	10.36 vs 13.50	0.183	10.11 vs 13.94
Normal sporadic vs BRCA tissue without p53 accumulation vs BRCA tissue with p53 accumulation vs FTC	0.177	18.31 vs 26.08 vs 24.57 vs 31.88	0.082	16.69 vs 26.62 vs 25.50 vs 32.00	0.027	15.50 vs 27.77 vs 24.86 vs 33.19

CMI_{all}, cumulative methylation index of all genes; CMI_{hyper}, CMI of only the genes passing the hypermethylation threshold of 15%; CMI_{freq}, CMI of frequently hypermethylated genes ($\geq$20% of cases); FTC, fallopian tube carcinoma.

SFRP1, PTEN, PAX6, MUS81, APAF1, DNAJC15, PCCA, NF1 and *PCNA*.

Fifteen of these genes (in bold) were also frequently methylated in normal fallopian tube tissue.

Discussion

The purpose of this study was to investigate promoter methylation levels of a group of tumour suppressor genes in fallopian tubes of women with and without a *BRCA* germ line mutation and in FTC. Besides looking at individual genes, we assessed overall methylation levels by the CMI as before. Fackler *et al*. (2004) were probably the first to describe CMI as an overall measure of methylation of the genes that were part of a quantitative multiplex methylation-specific PCR panel. Since then, their group has used CMI to detect tumour cells in nipple fluid (Fackler *et al*. 2006), nipple discharge (Fackler *et al*. 2009) and serum (Kim *et al*. 2010*a*) to assess methylation levels in familial breast cancer (Swift-Scanlan *et al*. 2011), different breast cancer subtypes (Bae *et al*. 2004, Lee *et al*. 2010), breast cancer in different ethnic groups (Lee *et al*. 2007*a*), ductal carcinoma *in situ* of the breast (Lee *et al*. 2008) and cervical cancer in liquid-based cytology (Kim *et al*. 2010*b*). We have also used CMI in our own previous studies. We studied methylation levels in sporadic and hereditary breast cancer using the CMI (Suijkerbuijk *et al*. 2008) and found the CMI to be higher in normal breast epithelium from *BRCA* mutation carriers compared with non-mutation carriers, higher in ER-negative and lymph node-positive breast cancer and in sporadic compared with hereditary breast cancer. However, we did not find differences in CMI between sporadic and hereditary ovarian cancers (Bol *et al*. 2010). We also found higher CMI in endometrioid endometrial cancer compared with serous endometrial cancer and negative prognostic impact of high CMI (Seeber *et al*. 2010).

Our results show increased promoter methylation levels of tumour suppressor genes in fallopian tube tissue of *BRCA* mutation carriers compared with tissue of non-*BRCA* mutation carriers of a similar age. This is in line with a study by Locke *et al*. (2007), who found significantly higher methylation levels in normal breast cells from *BRCA* mutation carriers acquired through ductal lavage compared with *BRCA*-negative women, and with a study by Suijkerbuijk *et al*. (2008) who found a higher CMI in normal breast epithelium of *BRCA1* mutation carriers compared with non-*BRCA* mutation carriers. We are the first to report on this association in fallopian tube tissue.

No significant differences in methylation were found between areas with and without p53 accumulation in *BRCA* mutation carriers, both showed methylation levels between sporadic control tissue and FTC. Therefore, although *BRCA* mutations seem to lead to higher baseline methylation levels in normal fallopian tube tissue, areas with p53 accumulation seem no more likely than control epithelium to harbour methylated genes.

A number of normal *BRCA* and non-*BRCA* ovaries were studied to investigate whether the difference in methylation levels between *BRCA* and non-*BRCA* mutation carriers could also be observed in a different

tissue type. Although the sample size was too small to draw firm conclusions, the *BRCA* ovaries show an overall higher methylation level compared with non-*BRCA* ovaries, which is similar to our observations in fallopian tube tissue. The methylation patterns of normal ovary and fallopian tube show a fairly high degree of overlap, with over 50% of methylated genes being identical in both tissues. Interestingly, the baseline methylation levels in both *BRCA* and non-*BRCA* ovaries seem to be higher than those in the fallopian tube.

In all our analyses, methylation levels of tumour suppressor genes in FTC were higher than those found in both sporadic and *BRCA* benign fallopian tube tissue; FTC thus joins the ranks of numerous cancer types showing hypermethylation of tumour suppressor genes, including breast, lung and colon cancer (Esteller 2008).

A comparison of the literature shows that the methylation patterns found in FTC share some similarities with those in ovarian cancer. *RASSF1* (Makarla *et al*. 2005, Choi *et al*. 2006, Bol *et al*. 2010), *APC* (Rathi *et al*. 2002, Makarla *et al*. 2005), *CDH13* (Makarla *et al*. 2005, Bol *et al*. 2010), *CDH1* (Rathi *et al*. 2002) and *GSTP1* (Makarla *et al*. 2005, Bol *et al*. 2010), which showed an increased methylation frequency in FTC compared with control tissue in this study, have previously been reported in ovarian cancer and not in its precursors. Choi *et al*. (2006) showed that *RASSF1* was frequently methylated in borderline tumours and ovarian carcinoma and showed a significant correlation with the presence of peritoneal implants, micro-invasion and bilaterality. Interestingly, four genes that were only found in invasive ovarian cancer and not in non-invasive lesions or normal tissue, *RASSF1, APC, MGMT* and *GSTP1* (Makarla *et al*. 2005), showed exactly the same pattern in this study. Methylation of these genes could, therefore, play a role in the progression from non-invasive to invasive cancer. *H2AFX*, which was specific for FTC but not for ovarian cancer, is involved in DNA damage repair and has been implicated in several other cancer types (Warters *et al*. 2005, Brunner *et al*. 2011).

The above stated evidence may point to a common origin of FTC and serous ovarian cancer as previously proposed (Piek *et al*. 2003, 2007, Przybycin *et al*. 2010, Mehra *et al*. 2011, Seidman *et al*. 2011).

These methylated genes are partly linked. *RASSF1* (isoform A) has been shown to be able to inhibit *APC* through the inhibition of Cdc20 (Song & Lim 2004). In normal conditions, Cdc20 binds directly to *APC* (forming the *APC*–Cdc20 complex), which is crucial for *APC* activation. Alterations in this pathway are able to accelerate mitotic cell progression, a feature commonly observed in cancer. *RASFF1a* also prevents degradation of p53 by increasing self-ubiquitination of MDM2, a protein necessary for p53 degradation (Song *et al*. 2008). *TP53* (of which p53 is the gene product) was not frequently methylated in our study; however, mutations in *TP53* are a common feature in both FTC and high-grade serous ovarian cancer (in over 80% of cases; Kurman *et al*. 2008) and upregulation of p53 by immunohistochemistry is frequently observed. *TP73* encodes a member of the p53 family of transcription factors. Certain isoforms lacking a transactivation domain may be able to block the function of p53, the exact mechanism by which this occurs is, however, not yet understood. Methylation of *RASSF1a* and *TP73* could, therefore, theoretically compromise *APC* and p53 function and thereby contribute to carcinogenesis.

The functional meaning of the methylation found needs to be established, since there is no one-on-one correlation between promoter methylation of a single CpG island and gene expression (Gort *et al*. 2008). Functionally, several changes have been described that may accompany increased methylation levels, without a proven causal relationship. Piek *et al*. (2001) described that morphologically normal tubal epithelium of *BRCA* mutation carriers contained a higher proportion of ki67-expressing cells and lower fractions of cells expressing p21 and p27 than controls.

We also compared results from this study and studies on breast (Suijkerbuijk *et al*. 2008), ovarian (Bol *et al*. 2010) and endometrial cancer (Seeber *et al*. 2010), which also utilised MS-MLPA and the CMI. *RASSF1* showed frequent methylation in all cancer types. Ovarian cancer and FTC showed overlap for three out of four frequently methylated genes (*RASSF1, CDH13* and *GSTP1*) as did endometrial cancer and

Table 5 Comparison of frequently hypermethylated genes between studies (indicated by +)

	This study: methylation in FTC	Bol *et al.* (2010): methylation in ovarian cancer	Suijkerbuijk *et al.* (2008): methylation in breast cancer	Seeber *et al.* (2010): methylation in endometrial cancer
RASSF1	+	+	+	+
APC	+			+
GSTP1	+	+		
CDH13	+	+		+
TP73				+
MLH1				+
TIMP2				+

FTC (*RASSF1, CDH13* and *APC*). Breast cancer and FTC only shared frequent methylation of *RASSF1* (Table 5).

The studied group of patients with FTC consisted solely of women without a confirmed *BRCA* germ line mutation. Whether there is a difference in methylation levels between *BRCA* and non-*BRCA* FTC is unknown and needs to be studied. Breast cancer studies have suggested that promoter methylation may play a smaller role in *BRCA*-associated breast cancer than in sporadic breast cancer (Suijkerbuijk *et al*. 2008, Vasilatos *et al*. 2009). However, a methylation study comparing *BRCA*-associated and sporadic ovarian cancer showed no difference between the groups (Bol *et al*. 2010), so in ovarian cancer, methylation seems to play an equally important role in *BRCA*-related and sporadic ovarian cancer.

An age-related increase in methylation levels has been described in several tissue types (Ahuja *et al*. 1998, Kwabi-Addo *et al*. 2007). Whether this is also true for fallopian tube tissue is unknown. For non-*BRCA* control tissue and *BRCA* tissue, we have nevertheless corrected for this possible effect by age matching these groups.

We have not addressed the question whether the methylation levels found lead to reduced protein expression, since the relationship between methylation and expression is a complicated one. First, the relationship between promoter methylation and gene expression is certainly not one-on-one, which is illustrated by Twist, for which there is no relationship between promoter methylation and expression (Gort *et al*. 2008). Secondly, for most genes, we just studied one CpG island, while there may be many more CpG islands in the promoter regions of the studied genes, which could affect transcription. Thirdly, the methylation levels of these CpG islands vary over a continuous scale, while there is unlikely a fixed threshold where transcription gets affected and protein expression is reduced. Studying the relationship between promoter methylation and expression would, therefore, require extensive studies on different CpG islands with various methylation levels and reliable methods to quantify expression differences (beyond immunohistochemistry that is rather a qualitative than quantitative method).

In conclusion, *BRCA* mutation carriers show increased hypermethylation of tumour suppressor genes in their non-malignant fallopian tube epithelium, closer to methylation levels in FTC than to normal sporadic tissue. A *BRCA* germ line mutation thus seems to predispose to hypermethylation of tumour suppressor genes, which may be a contributing factor in their increased risk of developing serous gynaecological malignancies.

Methylation patterns of FTC, described here for the first time, show resemblance to those previously described in ovarian carcinoma, implying a common origin of both entities.

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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