Promoter hypermethylation patterns in fallopian tube epithelium of \textit{BRCA1} and \textit{BRCA2} germ line mutation carriers

Jonathan G Bijron\textsuperscript{1,2}, Petra van der Groep\textsuperscript{1,3}, Eleonora B van Dorst\textsuperscript{2}, Laura M S Seeber\textsuperscript{1,2}, Daisy M D S Sie-Go\textsuperscript{1}, René H M Verheijen\textsuperscript{2} and Paul J van Diest\textsuperscript{1}

\textsuperscript{1}Department of Pathology, \textsuperscript{2}Division of Woman and Baby, Gynaecological Oncology and \textsuperscript{3}Division of Internal Medicine and Dermatology, University Medical Centre Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands

(Correspondence should be addressed to P J van Diest; Email: p.j.vandiest@umcutrecht.nl)

Abstract

\textit{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, intraepithelial 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 \textit{BRCA} mutation carriers. Cells from these areas were harvested together with cells from adjacent benign appearing areas. An age-matched non-\textit{BRCA} sporadic control group (\textit{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 \textit{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 \textit{BRCA} mutation carriers no significant differences were found. In this paper, we have shown that \textit{BRCA} mutation carriers display increased methylation of tumour suppressor genes in their nonmalignant 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 \textit{BRCA} mutation carriers.

Introduction

\textit{BRCA} mutation carriers are at hereditary high risk of developing serous ovarian cancer. For \textit{BRCA1} mutation carriers, the average cumulative risk to develop ovarian cancer by age 70 is 39\% (confidence interval (CI): 18–54\%) and for \textit{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 intraepithelial carcinoma) arising from the fallopian tubes, which is an established precursor of FTC and probably also serous ovarian carcinoma, is also more common in \textit{BRCA} mutation carriers (Piek et al. 2001, Hermsen et al. 2006).

These dysplastic lesions share a high rate of \textit{TP53} mutations with serous invasive carcinoma and often (79–100\%) show p53 accumulation by immunohistochemistry (Lee et al. 2007b, Shaw et al. 2009; Fig. 1). Recently, another lesion arising in the fallopian tube, the p53 signature, has been hypothesised to be an even
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. 2007b). 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 adeneectomy 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% H2O2 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. 2007b). 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 (≥15%) and frequently methylated genes (in ≥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

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP73</td>
<td>Tumour protein p73</td>
</tr>
<tr>
<td>CASP8</td>
<td>Caspase 8, apoptosis-related cysteine peptidase</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel–Lindau tumour suppressor</td>
</tr>
<tr>
<td>RARα</td>
<td>Retinoic acid receptor, beta</td>
</tr>
<tr>
<td>MLH1</td>
<td>mutL (Escherichia coli (E. coli)) homologue 1 (colon cancer, non-polyposis type 2)</td>
</tr>
<tr>
<td>RASSF1</td>
<td>Ras association (RalGDS/AF-6) domain family member 1</td>
</tr>
<tr>
<td>FHIT</td>
<td>Fragile histidine triad gene</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>ESR1</td>
<td>Oestrogen receptor 1</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CDKN2B</td>
<td>Cyclin-dependent kinase inhibitor 2B</td>
</tr>
<tr>
<td>DAPK1</td>
<td>Death-associated protein kinase 1</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>CD44</td>
<td>CD44 molecule (Indian blood group)</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Glutathione S-transferase pi 1</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>IGSF4</td>
<td>Immunoglobulin superfamily 4</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>Cyclin-dependent kinase inhibitor 1B</td>
</tr>
<tr>
<td>CHFR</td>
<td>Checkpoint with forkhead and ring finger domains</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer 2, early onset</td>
</tr>
<tr>
<td>CDH13</td>
<td>Cadherin 13, H-cadherin</td>
</tr>
<tr>
<td>HIC1</td>
<td>Hypermethylated in cancer 1</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1, early onset</td>
</tr>
<tr>
<td>TIMP3</td>
<td>Tissue inhibitor of metallopeptidase 3</td>
</tr>
<tr>
<td>MSH6</td>
<td>mutS homologue 6 (E. coli)</td>
</tr>
<tr>
<td>PAX5</td>
<td>Paired box 5</td>
</tr>
<tr>
<td>MGMT</td>
<td>O-6-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>PAX6</td>
<td>Paired box 6</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms tumour 1</td>
</tr>
<tr>
<td>CADM1</td>
<td>Cell adhesion molecule 1</td>
</tr>
<tr>
<td>RB1</td>
<td>Retinoblastoma 1</td>
</tr>
<tr>
<td>THBS1</td>
<td>Thrombospondin 1</td>
</tr>
<tr>
<td>PYCARD</td>
<td>PYD and CARD domain containing</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumour protein p53</td>
</tr>
<tr>
<td>STK11</td>
<td>Serine/threonine kinase 11</td>
</tr>
<tr>
<td>GATA5</td>
<td>GATA-binding protein 5</td>
</tr>
<tr>
<td>PRDM2</td>
<td>PR domain containing 2</td>
</tr>
<tr>
<td>RUNX3</td>
<td>Runt-related transcription factor 3</td>
</tr>
<tr>
<td>HLTF</td>
<td>Helicase-like transcription factor</td>
</tr>
<tr>
<td>SCGB3A1</td>
<td>Secretoglobin, family 3A, member 1</td>
</tr>
<tr>
<td>ID4</td>
<td>Inhibitor of DNA binding 4</td>
</tr>
<tr>
<td>Twist1</td>
<td>Twist homologue 1 (Drosophila)</td>
</tr>
<tr>
<td>SFRP4</td>
<td>Secreted frizzled-related protein 4</td>
</tr>
<tr>
<td>DLC1</td>
<td>Deleted in liver cancer</td>
</tr>
<tr>
<td>SFRP5</td>
<td>Secreted frizzled-related protein 5</td>
</tr>
<tr>
<td>H2AFX</td>
<td>H2A histone family, member X</td>
</tr>
<tr>
<td>CCND2</td>
<td>Cyclin D2</td>
</tr>
<tr>
<td>CACNA1G</td>
<td>Calcium channel, voltage-dependent, T-type, alpha 1G subunit</td>
</tr>
<tr>
<td>TGIF</td>
<td>TGFB-induced factor homeobox</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>CACNA1A</td>
<td>Calcium channel, voltage-dependent, P/Q-type, alpha 1A subunit</td>
</tr>
</tbody>
</table>

Table 1 continued

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPHB2</td>
<td>EPH receptor B2</td>
</tr>
<tr>
<td>LMNA</td>
<td>Lamin A/C</td>
</tr>
<tr>
<td>NTRK1</td>
<td>Neurotrophic tyrosine kinase receptor, type 1</td>
</tr>
<tr>
<td>RARRES1</td>
<td>Retinoid acid receptor responder (tazarotene induced)</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
</tr>
<tr>
<td>IGF2R</td>
<td>Insulin-like growth factor 2 receptor</td>
</tr>
<tr>
<td>GATA4</td>
<td>GATA-binding protein 4</td>
</tr>
<tr>
<td>SFRP1</td>
<td>Secreted frizzled-related protein 1</td>
</tr>
<tr>
<td>M1N1</td>
<td>Multiple endocrine neoplasia 1</td>
</tr>
<tr>
<td>MUS81</td>
<td>MUS81 endonuclease homologue (Saccharomyces cerevisiae)</td>
</tr>
<tr>
<td>RBM14</td>
<td>RNA-binding motif protein 1</td>
</tr>
<tr>
<td>WIFI</td>
<td>WNT inhibitory factor 1</td>
</tr>
<tr>
<td>APAF1</td>
<td>Apoptotic peptidase activating factor 1</td>
</tr>
<tr>
<td>DNAJC15</td>
<td>DnaJ (Hsp40) homologue, subfamily C, member 15</td>
</tr>
<tr>
<td>PCCA</td>
<td>Propionyl coenzyme A carboxylase, alpha polypeptide</td>
</tr>
<tr>
<td>CDH1</td>
<td>Cadherin 1, type 1, E-cadherin (epithelial)</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromin 1</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PXMP4</td>
<td>Peroxisomal membrane protein 4</td>
</tr>
</tbody>
</table>

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 WIFI, 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 +)

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

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, HLT, SCGB3A1, SFRP4, DLC1 (2×, with different probes), SFRP5 (2×, with different probes), H2AFX, CANA1G, BCL2, CACNA1A, IGF2R, MEN1, RBM14, APAF1 (2×, with different probes), DNAJC15, CDH1, NF1, PCNA and PXMP4). RB1, STK11, RUNX3, PAX6 and WIFI 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

<table>
<thead>
<tr>
<th>Gene/probe</th>
<th>Group a vs Group b</th>
<th>Group a vs Group c</th>
<th>Group a vs Group d</th>
<th>Group b vs Group c</th>
<th>Group b vs Group d</th>
<th>Group c vs Group d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean rank</td>
<td>P value</td>
<td>Mean rank</td>
<td>P value</td>
<td>Mean rank</td>
<td>P value</td>
</tr>
<tr>
<td>CASP8_2761</td>
<td>10.42 vs 17.32</td>
<td>0.016</td>
<td>10.77 vs 17.00</td>
<td>0.028</td>
<td>7.31 vs 17.00</td>
<td>0.000</td>
</tr>
<tr>
<td>VHL_3810</td>
<td>10.88 vs 16.89</td>
<td>0.034</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RARβ_4040</td>
<td>8.65 vs 14.81</td>
<td>0.024</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APC_1905</td>
<td>8.65 vs 14.81</td>
<td>0.023</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44_3817</td>
<td>8.35 vs 15.31</td>
<td>0.010</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTP1_1638</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATM_4044</td>
<td>10.77 vs 17.00</td>
<td>0.033</td>
<td>10.15 vs 17.57</td>
<td>0.012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGSF4_3819</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP3_2255</td>
<td>10.85 vs 16.93</td>
<td>0.015</td>
<td>10.62 vs 17.14</td>
<td>0.023</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDKN1B_7949</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP73_1684</td>
<td>10.85 vs 16.93</td>
<td>0.046</td>
<td>10.65 vs 17.11</td>
<td>0.034</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSH6_1250</td>
<td>9.42 vs 18.25</td>
<td>0.004</td>
<td>10.50 vs 17.25</td>
<td>0.027</td>
<td>7.77 vs 16.25</td>
<td>0.002</td>
</tr>
<tr>
<td>VHL_3818</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDKN2A_1530</td>
<td>9.04 vs 18.61</td>
<td>0.002</td>
<td>10.73 vs 17.04</td>
<td>0.039</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGMT_5670</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIST1_2174</td>
<td>9.96 vs 17.75</td>
<td>0.0009</td>
<td>10.88 vs 16.89</td>
<td>0.043</td>
<td>8.58 vs 14.94</td>
<td>0.019</td>
</tr>
<tr>
<td>CHFR_2737</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCA2_2285</td>
<td>10.38 vs 17.36</td>
<td>0.022</td>
<td>7.92 vs 16.00</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RB1_2734</td>
<td>9.46 vs 17.54</td>
<td>0.006</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RB1_4502</td>
<td>14.19 vs 5.81</td>
<td>0.003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>THBS1_1678</td>
<td>8.73 vs 18.89</td>
<td>0.001</td>
<td>9.15 vs 14.00</td>
<td>0.048</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PYCARD_2252</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDH1_2257</td>
<td>10.77 vs 17.00</td>
<td>0.041</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STK1_6783</td>
<td>12.38 vs 8.75</td>
<td>0.044</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RUNX3_11131</td>
<td>13.08 vs 7.62</td>
<td>0.050</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLTF_2758</td>
<td>10.96 vs 16.82</td>
<td>0.038</td>
<td>7.77 vs 16.25</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCG3A1_3005</td>
<td>11.15 vs 16.64</td>
<td>0.038</td>
<td>8.73 vs 14.89</td>
<td>0.029</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFRP4_3744</td>
<td>10.23 vs 17.50</td>
<td>0.013</td>
<td>7.65 vs 16.44</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFRP5_9149</td>
<td>7.65 vs 16.44</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2AFX_8509</td>
<td>8.27 vs 15.44</td>
<td>0.007</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CACNA1G_10123</td>
<td>8.54 vs 15.00</td>
<td>0.020</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2AFX_8509</td>
<td>8.27 vs 15.44</td>
<td>0.007</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFRP5_9148</td>
<td>7.77 vs 16.25</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2AFX_8509</td>
<td>8.27 vs 15.44</td>
<td>0.007</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFRP5_9148</td>
<td>8.32 vs 17.06</td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2AFX_8509</td>
<td>8.27 vs 15.44</td>
<td>0.007</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFRP5_9148</td>
<td>8.32 vs 17.06</td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2AFX_8509</td>
<td>8.27 vs 15.44</td>
<td>0.007</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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,
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., 2010a) 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., 2007a), ductal carcinoma *in situ* of the breast (Lee et al., 2008) and cervical cancer in liquid-based cytology (Kim et al., 2010b). 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 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 appear 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 breast cancer (Table 5).

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

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RASSF1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>APC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>GSTP1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CDH13</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>TP73</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MLH1</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>TIMP2</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
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 (Suikerkbuijk 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.

Funding

This work was supported by ‘De Hendrik Muller Stichting’, ‘Het Vreedefonds’, ‘Stichting de Fundatie van de Vrijvrouwe van Renswoude’ and ‘Studiefonds Ketel 1’. L M S Seeber was supported by the Dutch Cancer Society Project 2009-4375.

Acknowledgements

The authors thank Mr Remco Radersma for technical advice and Cathy Moelans, PhD, for performing additional analyses. They also thank Doug Lin for helping with study materials.

References


Callahan MJ, Crum CP, Medeiros F, Kindelberger DW, Elvin JA, Garber JE, Feltmate CM, Berkowitz RS & Muto MG 2007 Primary fallopian tube malignancies in...


Gort EH, Súijkerbuijk KP, Roothaan SM, Vooijs M, van der Wall E & van Diest PJ 2008 Methylation of the TWIST1 promoter, TWIST1 mRNA levels, and immunohistochemical expression of TWIST1 in breast cancer. *Cancer Epidemiology, Biomarkers & Prevention* **17** 3325–3330. (doi:10.1158/1055-9965.EPI-08-0472)


Lee JS, Fackler MJ, Teo WW, Lee JH, Argani P & Sukumar S 2008 Quantitative promoter hypermethylation...


Song MS & Lim DS 2004 Control of APC–Cdc20 by the tumor suppressor RASSF1A. *Cell Cycle* 3 574–576. (doi:10.4161/cc.3.5.887)


Received in final form 7 November 2011
Accepted 2 December 2011
Made available online as an Accepted Preprint 5 December 2011