Methylation profiles of endometrioid and serous endometrial cancers

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Abstract

Promoter methylation is a gene- and cancer type-specific epigenetic event that plays an important role in tumour development. As endometrioid (endometrioid endometrial carcinoma, EEC) and serous endometrial cancers (uterine papillary serous carcinoma, UPSC) exhibit different clinical, histological and molecular genetic characteristics, we hypothesized that these differences may be reflected in epigenetic phenomena as well. Identification of a panel of methylation biomarkers could be helpful in a correct histological classification of these two subtypes, which solely on the basis of morphology is not always easy. Methylation-specific multiplex ligation-dependent probe amplification was used to assess the extent of promoter methylation of different tumour suppressor genes in EEC and UPSC. Methylation results were correlated with histology and survival. The median cumulative methylation index of all genes was significantly higher in EEC (124) than in UPSC (93) ($P < 0.001$). Promoter methylation of CDH13 and MLH1 was more frequently present in EEC, while CDKN2B and TP73 were more frequently methylated in UPSC. Almost 90% of EEC and 70% of UPSC could be predicted by CDH13 and TP73. In EEC, methylation of MLH1 was associated with a shorter disease-free survival (DFS; $P < 0.0001$) and overall survival (OS; $P = 0.005$). In a multivariate model, MLH1 methylation emerged as an additional prognostic factor to stage for DFS ($P = 0.002$). In conclusion, promoter methylation is more common in EEC than UPSC. A panel of methylation biomarkers could be useful to distinguish between the two histological subtypes of endometrial cancer. Furthermore, methylation of MLH1 may have prognostic value in EEC.

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Introduction

Tumours presumably develop by a stepwise accumulation of interacting epigenetic and genetic events over time (Baylin & Herman 2000, Jones & Baylin 2002). Promoter methylation is an epigenetic event that plays an important role in both normal and tumour cells. In normal cells, it contributes to chromatin organization, silencing of transposable elements, X-chromosome inactivation, tissue-specific expression and genetic imprinting. In cancer cells, besides global

hypomethylation of the genome, methylation of CpG islands occurs (Baylin & Herman 2000). These CpG islands are cytosine- and guanine-rich areas in the promoter regions of genes that are involved in cell cycle, cell adherence, DNA repair and apoptosis. Methylation of the CpG islands leads to inactivation of these genes (Baylin & Herman 2000, Jones & Baylin 2002). CpG island methylation is gene-specific and cancer type-specific (Costello *et al.* 2000, Yang *et al.* 2006, Esteller 2008). Specific methylation patterns

may provide a useful signature for tumour diagnosis and may be used to assess prognosis.

In the Western world, endometrial cancer is the most common malignant tumour of the female genital tract. The American Cancer Society estimates that 42 160 women will have been diagnosed with, and 7780 women will have died of endometrial cancer in 2009 in the USA (Jemal *et al.* 2009). In the endometrium, different subtypes of cancer can develop. Endometrioid endometrial carcinoma (EEC), or type 1 cancer, accounts for ~75% of cases. These tumours are oestrogen-dependent and tend to be of lower grade, and have fewer recurrences and a better survival than other types. They often develop in a background of atypical complex hyperplasia and are sometimes characterized by mutations in phosphatase and tensin homologue (PTEN) and defects in DNA mismatch repair – as manifested by microsatellite instability (MSI). Ninety percentage of EEC cases are sporadic, while the remaining 10% are hereditary. For example, germ line mutations in the mismatch repair genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*) result in Lynch syndrome, a familial syndrome associated with elevated risk of colorectal cancer and EEC (Bocker *et al.* 1999). Type 2 tumours, of which uterine papillary serous carcinoma (UPSC) is the most common subtype, arise from atrophic endometrium in older women. Its precursor lesion endometrial intraepithelial carcinoma (EIC) also differs as compared with EEC. These type 2 tumours are often poorly differentiated, and have a greater propensity for early spread and a worse prognosis than EEC. So far, they have not been associated with a hereditary tumour syndrome. Type 2 tumours often contain p53 mutations and are usually non-diploid. Although these specific genetic alterations have been described in endometrial cancers, none of these changes occur in a majority of cases. UPSC is rare, mixed patterns – defined as UPSC with a component of adenocarcinoma – do occur (Sherman *et al.* 1992), and diagnosis of UPSC, based on histology and p53 immunohistochemistry, may therefore be difficult. Moreover, some EEC does show a villoglandular and even papillary growth pattern that may mimic UPSC. Since classification as EEC and UPSC has therapeutic and prognostic implications, it is important to make the proper diagnosis. In view of the described differences in clinical, histological and molecular genetic characteristics of EEC and UPSC, we hypothesized that these differences also extend to methylation patterns. In EEC, methylation of *MLH1* (Esteller *et al.* 1999, Horowitz *et al.* 2002, Kanaya *et al.* 2003), *RARB2* (Arafa *et al.* 2008), *RASSF1* (Arafa *et al.* 2008) and *CDKN2A* (*p16*) (Guida *et al.* 2009) has been suggested

as early events in carcinogenesis. Many other epigenetically silenced genes have been described in EEC, such as *MGMT* (Furlan *et al.* 2006), *PTEN* (Salvesen *et al.* 2001, Macdonald *et al.* 2004) and *GSTP1* (Chan *et al.* 2005). However, little is yet known about the role of methylation in UPSC (Xiong *et al.* 2005, Xie *et al.* 2007). Establishment of methylation profiles could improve our understanding of carcinogenesis in both subtypes of endometrial cancer and may lead to identification of a panel of methylation biomarkers, which can be used as an additional tool for correct classification of these two histological subtypes. Therefore, the aim of our study was to compare methylation status of a set of common tumour suppressor genes (TSGs) in EEC and UPSC, in order to arrive at a methylation signature of EEC and UPSC, and to evaluate prognostic value of methylation.

Materials and methods

Patients and tissues

Paraffin-embedded specimens from EEC ($n=93$) and UPSC ($n=26$) were selected from the archives of the Department of Pathology of the University Medical Centre, Utrecht, The Netherlands, with additional samples of UPSC from the University Medical Centre Nijmegen, Leiden University Medical Centre and the VU University Medical Centre Amsterdam. Only patients with hysterectomy samples available were included. Furthermore, patients were excluded if they had a second primary tumour of the cervix or ovary, or a history of cervical carcinoma. None of the patients received preoperative radio- or chemotherapy. Haematoxylin- and eosin-stained sections were revised and histologically typed and graded by two pathologists. The tumour stage and grade were defined by the International Federation of Gynaecology and Obstetrics (FIGO) system (Creasman *et al.* 2006). Histological grading only applied to EEC; UPSC is considered high grade by definition. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our network hospitals (van Diest 2002).

Table 1 gives an overview of the baseline clinicopathological features. There was a significant correlation between tumour type and p53 positivity ($P<0.0001$), with p53 being positive in 21% of EEC and in 62% of UPSC.

Survival analysis was restricted to EEC since there were too few UPSC cases for meaningful statistics. In EEC, three patients with unknown recurrence status were censored for disease-free survival (DFS) analysis. Locoregional recurrences (vaginal, pelvic or both) or

Table 1 Patient demographics and main pathological features of the study group of endometrial cancer patients ($n=119$)

Variable	Grouping	N (%)
Endometrioid ($n=93$)		
Age	Minimum	40
	Maximum	88
	Mean	63
Stage	I	55 (59)
	II	22 (24)
	III	11 (12)
	IV	5 (5)
Grade	1	28 (30)
	2	46 (50)
	3	19 (20)
Grade 2nd revision	1	56 (47)
	2	10 (8)
	3	27 (25)
Depth of myometrial invasion	0	3 (3)
	<50%	49 (53)
	>50%	41 (44)
Recurrence/metastasis	Yes	17 (19)
	No	73 (75)
	Missing	3 (6)
Survival	Alive	68 (73)
	Death of endometrial cancer	10 (11)
	Death of intercurrent disease	11 (12)
	Cause of death unknown	3 (3)
	Missing	1 (1)
Serous ($n=26$) ^a		
Age	Minimum	57
	Maximum	88
	Mean	69
Stage ^b	I	8 (31)
	II	7 (27)
	III	7 (27)
	IV	3 (11)
Depth of myometrial invasion ^b	0	1 (4)
	<50%	6 (23)
	>50%	18 (69)

^aSerous endometrial carcinoma is considered high grade by definition.

^bStage and MI missing for $n=1$.

distant metastases were found in 17 of 90 patients. The follow-up was 12–182 months (mean 72 months) for surviving patients.

Immunohistochemistry

Immunohistochemistry was performed on 4 μ m-thick paraffin slides. Slides were deparaffinized with xylene and serial ethanol dilutions, and endogenous peroxidase activity was blocked in a buffer solution of pH 5.8 with hydrogen peroxide followed by antigen retrieval. Antigen retrieval was performed with citrate buffer, pH 6.0, for 20 min at boiling temperature.

After a cooling down period of 20 min, the slides were incubated with the primary p53 antibody (DakoCytomation, Glostrup, Denmark) for 1 h at room temperature, followed by the secondary antibody (Poversion, ImmunoVision Technologies, Brisbane, CA, USA; ready to use, 30 min). Slides were developed with diaminobenzidine for 10 min, followed by haematoxylin counterstaining. In between steps, slides were washed in PBS. Before the slides were mounted, all sections were dehydrated in alcohol and xylene. Appropriate positive and negative controls were used throughout.

Evaluation of staining

Two authors (P v D and L S) scored all slides blinded to clinico-pathological data and promoter methylation results. The percentage of dark, homogenously stained nuclei was estimated, ignoring wild-type staining. p53 nuclear staining in more than 10% of cells was considered as positive (Fig. 1). Staining was evaluated through a standard light microscope (Leica DM6000B) at 200–400 $\times$ final magnification. Pictures were taken with a Leica digital camera DMX1200 through a 10 $\times$ objective.

DNA extraction

Paraffin sections of 4 μ m were cut, placed on slides and dried overnight. After deparaffinization, mesodissection of the relevant tissue was performed with a scalpel on the basis of marked haematoxylin- and eosin-stained serial sections. The tissue was suspended in 100 μ l lysis buffer (50 mM Tris-HCl, pH 8.0; 0.5%

Figure 1 Positive p53 nuclear immunohistochemical staining in serous endometrial cancer tissue. Positive staining in more than 10% of cells was considered as positive.

Tween 20). Two hundred milligrams of proteinase K (Sigma) were added. After 1 h incubation at 56 °C, samples were heat inactivated for 10 min at 99 °C and immediately placed on ice. Samples were subsequently centrifuged for 2 min at 14 000 r.p.m. (22 000 g), after which the supernatant was stored at 4 °C.

Methylation-specific multiplex ligation-dependent probe amplification

The ME001 methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) probe mix (MRC Holland, Amsterdam, The Netherlands) was used to study promoter methylation of 24 TSGs (see Table 2 for list of genes). These TSGs are known to be frequently silenced by methylation in different tumours. The principle of the MS-MLPA technique has been described elsewhere (Nygren et al. 2005). The experimental procedure followed the recommendations of the manufacturer. Reaction products were separated by capillary electrophoresis (on an ABI 310 automated DNA sequencer, Applied Biosystems, Foster City, CA, USA) and analysed using GeneScan genotyping software (Applied Biosystems). The method is based on probes that target CpG islands within the promoter regions of the TSGs. The probes

contain a recognition site for the methylation-sensitive restriction enzyme HhaI. HhaI digests unmethylated DNA but leaves methylated sites intact, generating a signal if DNA is undigested. The peak area of each probe was normalized by dividing it by the combined areas of the control probes. Each normalized peak area from a digested sample was compared with that obtained in the undigested sample. The peak sizes and areas were exported to an Excel file, and the normalized areas from the digested and undigested samples were compared to determine the methylation dosage ratio. This is described by the following calculation: $D_m = (P_x/P_{ctrl})_{Dig}/(P_x/P_{ctrl})_{Lig}$, where D_m is the methylation dosage ratio, P_x is the peak area of a given probe, P_{ctrl} is the sum of the peak areas of all control probes. Dig stands for HhaI digested sample and Lig for undigested sample. 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. All cases were analysed in duplicate, using the mean methylation percentage of the two runs in the statistical analysis. The cumulative methylation index (CMI) was calculated as the sum of the percentage methylation for all genes as before (Suijkerbuijk et al. 2008; Supplementary Figure 1, see section on supplementary data given at the end of this article).

Table 2 Tumour suppressor genes studied

Gene symbols	Gene names
APC	Adenomatous polyposis coli
ATM	Ataxia telangiectasia mutated
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
CASP8	Caspase 8
CD44	CD44 antigen precursor
CDH13	Cadherin 13, H-cadherin
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27 ^{kip})
CDKN2A	Cyclin-dependent kinase inhibitor 2A (encoding p14ARF)
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15)
CHFR	Checkpoint protein with forkhead and RING finger domains
DAPK1	Death-associated protein kinase 1
ESR1	Oestrogen receptor 1
FHIT	Fragile histidine triad gene
GSTP1	Glutathione S-transferase pi
HIC1	Hypermethylated in cancer 1
IGSF4	Immunoglobulin superfamily, member 4
MLH1	MutL homologue 1
PTEN	Phosphatase and tensin homologue
RARβ	Retinoic acid receptor, β
RASSF1A	RAS-associated domain family 1 (isoform A)
TIMP3	Tissue inhibitor of metalloproteinases 3
TP73	Cellular tumour antigen p73
VHL	Von Hippel–Lindau disease tumour suppressor

Statistical analysis

Mann–Whitney and Kruskal–Wallis tests were used for comparing medians between groups. The χ^2 -test was used for comparing proportions. Spearman correlation coefficients were computed for assessing the association between methylation and other continuous variables. Hierarchical clustering was applied using R and SPSS (Chicago, IL, USA). We performed logistic regression analysis to examine the predictive value of methylation pattern on tumour type, calculating 95% confidence intervals (CI) of the regression coefficients. Univariate survival analysis was restricted to EEC since there were too few UPSC cases for meaningful statistics. Kaplan–Meier curves were plotted, and differences between the curves were analysed with the log-rank test. The covariates stage, grade and myometrial invasion were next to methylation data used in multivariate analysis by Cox regression. Statistical tests were considered statistically significant at two-sided $P \leq 0.05$. SPSS 15.0 for Windows was used for statistical analysis.

Results

Overall frequencies of promoter methylation

Using a methylation dosage ratio of 0.15 as threshold, an average of three TSGs were methylated per tumour. There was a significant difference in the number of methylated genes between EEC (average 3.0 genes) and UPSC (average 2.0 genes) (*t*-test; $P=0.008$). In individual tumours, the number of methylated genes ranged from 0 to 7. Of the six tumours showing no methylation at all, five were UPSC.

Methylation patterns of individual genes

Aberrant methylation in at least 20% of the cases was observed for five genes in EEC (*TIMP3*, *APC*, *RASSF1*, *CDH13* and *MLH1*) and for four genes in UPSC (*CDKN2B*, *RASSF1*, *TP73* and *CDH13*). *CDH13* was most frequently methylated in 101/120 cases. *RASSF1* showed frequent methylation in both cancers. Among genes showing significant tumour-type specificity,

methylation of *TIMP3*, *CDH13* and *MLH1* was more frequently found in EEC. Interestingly, *MLH1* was never methylated in UPSC. Methylation of *CDKN2B* and *TP73* was not very frequent, but if present, it was quite typical of UPSC (27 vs 9% and 20 vs 4% respectively). Methylation of the frequently methylated genes was not correlated with stage. Only *CDH13* methylation was correlated with age; the mean age in the unmethylated cases was significantly higher ($P=0.016$). In the EEC cases, *CDH13* was significantly less frequently methylated in grade 3 tumours ($P=0.002$). In the PORTEC-1 trial (Scholten *et al.* 2004), the reproducibility of grade 2 was found to be poor; therefore, a second revision of grade was done (V S) (Table 1) Again, only a correlation between *CDH13* methylation and grade was found. When comparing grade 3 EEC and UPSC, *APC* and *MLH1* were significantly more often methylated in EEC, and *CDKN2B* was methylated in UPSC. Of the 24 TSGs, *ATM*, *HIC1*, *CDKN1B*, *BRCA2*, *DAPK1* and *VHL1* were never methylated in EEC or UPSC.

Table 3 Tumour suppressor gene methylation profiles in 119 endometrial cancers

Genes	Endometrioid			Serous		
	Freq meth cases (%)	Quantitative methylation levels in % (range)	Mean %	Freq meth cases (%)	Quantitative methylation levels in % (range)	Mean %
APC	29 (31)	15–83	47	5 (19)	24–69	42
<i>ATM</i>	–	–	–	–	–	–
<i>BRCA1</i>	3 (3)	31–48	37	1 (4)	22	22
<i>BRCA2</i>	–	–	–	–	–	–
<i>CASP8</i>	6 (6)	16–68	42	1 (4)	24	24
<i>CD44</i>	4 (4)	22–49	31	0	0	–
CDH13	87 (93)	16–118	55	13 (50)	15–81	44
<i>CDKN1B</i>	–	–	–	–	–	–
<i>CDKN2A</i>	2 (2)	16–18	18	–	–	–
CDKN2B	8 (9)	15–40	22	7 (27)	15–43	31
<i>CHFR</i>	8 (9)	26–109	58	–	–	–
<i>DAPK1</i>	–	–	–	–	–	–
<i>ESR1</i>	2 (2)	20–47	34	–	–	–
<i>FHIT</i>	2 (2)	46–89	68	–	–	–
<i>GSTP1</i>	14 (15)	0–58	25	–	–	–
<i>HIC1</i>	–	–	–	–	–	–
<i>IGSF4</i>	2 (2)	20–37	29	–	–	–
MLH1	24 (26)	14–132	65	–	–	–
<i>PTEN</i>	1 (1)	17	17	–	–	–
<i>RARB</i>	1 (1)	32	32	1 (4)	36	36
RASSF1A	74 (79)	16–90	51	20 (77)	16–91	48
TIMP3	25 (27)	15–89	38	2 (8)	19–51	35
TP73	4 (4)	15–19	17	5 (20)	15–19	18
<i>VHL1</i>	–	–	–	–	–	–
<i>CMI</i> (cumulative methylation index)		0–508	155		0–220	83

The bold genes are the frequently methylated genes.

Figure 2 Clustering of samples according to the methylation status of frequently methylated genes. The dendrogram is linked to the correlated histological subtype, grade, p53 status and age.

Quantitative methylation levels

The extent of methylation of the various genes is specified in Table 3. Overall, the quantitative methylation levels were higher in EEC than in UPSC. The CMI ranged from 0 to 508, and was significantly higher in EEC (median 124) than in UPSC (median 93; $P < 0.001$). CMI was also significantly higher in grade 3 EEC compared to UPSC. High CMI (> 124) was not correlated with stage, grade, age or p53 positivity. The individual methylation levels varied between the different TSGs. Of the frequently methylated genes, *MLH1* and *CDH13* showed highest quantitative methylation levels (Table 3). *CDKN2B* and *TP73* showed the lowest mean quantitative methylation levels (26 and 17% respectively) of those genes showing any methylation. Six genes were never methylated as stated above.

Clustering and regression analysis

Figure 2 shows clustering of the samples according to the methylation status of the frequently methylated genes. The dendrogram was linked to the correlated histological subtype, grade, p53 status and age. Unsupervised hierarchical clustering of the frequently methylated genes (*APC*, *CDH13*, *MLH1*, *RASSF1*, *TIMP3*, *CDKN2B* and *TP73*) segregated the tumours for the greater part into the histological subtypes. Clustering by p53 did not result in a specific pattern, nor did clustering by age at the time of diagnosis.

Figure 3 Clustering of samples according to the methylation status of all the 24 genes studied. Black bars correspond to methylated genes, white bars to unmethylated genes.

Methylation status seemed to cluster tumours best as per grade. Most of the grade 3 tumours clustered, irrespective of their endometrioid or serous background. Clustering of the samples according to the methylation status of all the 24 genes studied is shown in Fig. 3. The red bars correspond to methylated genes, and the green bars correspond to unmethylated genes. A subgroup of UPSC cases showed no methylation of *CDH13* and partly no methylation of *RASSF1A* cluster as group A. A second subgroup of UPSC cases did show methylation of these genes together with methylation of *CDKN2B* and/or *TP73* and clustered as group B.

Using logistic regression, *CDH13* and *TP73* were found to be the best predictors of EEC and UPSC histology. Almost 90% of EEC and 70% of UPSC could be predicted with *CDH13* (regression coefficient 12.4; 95% CI 4.19–36.89) and *TP73* (regression coefficient 0.07; 95% CI 0.017–0.35).

Figure 4 Of the frequently methylated genes, only *MLH1* was significantly associated with a shorter DFS ($P < 0.0001$) and OS ($P = 0.005$) in univariate analysis.

Survival analysis

In EEC, stage and grade showed prognostic value as expected. A CMI of 124 or more was correlated with a shorter disease-free survival (DFS) and overall survival (OS; $P = 0.004$ and $P = 0.009$ respectively). Of the frequently methylated genes, only *MLH1* was significantly associated with a shorter DFS ($P < 0.0001$) and OS ($P = 0.005$) in univariate analysis (Fig. 4). In a multivariate model together with stage, grade, depth of myometrial invasion and CMI, only *MLH1* methylation emerged as an additional prognostic factor to stage for DFS ($P = 0.002$). p53 expression was not correlated with survival.

Discussion

The aim of this study was to compare methylation status of some common TSGs to arrive at a methylation signature of EEC and UPSC that could be of additional value in the correct diagnosis of the two entities. Furthermore, their prognostic value was evaluated. The results indicate that the pattern of gene promoter methylation is indeed associated with the histological subtype of endometrial cancer. These particular characteristics can firstly give insight into the carcinogenetic pathways altered in these tumour types. Secondly, a panel of these methylation biomarkers could be useful to distinguish between EEC and UPSC in daily pathology practice, which is yet, based on histology and p53 immunohistochemistry, often not easy. Lastly, methylation status could be used for the assessment of prognosis of EEC. The fact that MS-MLPA requires only a small amount of DNA which can be paraffin derived is of great practical value to this end.

Overall methylation (as reflected by the CMI) was lower in UPSC, and of the six tumours showing no methylation at all, five were UPSC. This indicates that carcinogenesis of UPSC may be less dependent on promoter methylation, at least for the genes studied. The methylation of mammalian genomic DNA is catalyzed by DNA methyltransferases (DNMTs). Liao *et al.* (2008) identified two distinct DNMT1 protein expression patterns in endometrial carcinoma. Expression was significantly higher in EEC compared to UPSC. These results concur with observations by Xiong *et al.* (2005) that upregulation of DNMTs mRNA was detected in EEC, while downregulation of DNMTs occurred in UPSC. The lower overall methylation frequency and the lower quantitative methylation levels found in UPSC might be partly explained by this phenomenon.

Our results showed promoter methylation of *CDH13*, *MLH1* and *TIMP3* to be typical for EEC, while *CDKN2B* and *TP73* methylation characterized UPSC. Methylation of *CDH13* and *TP73* predicted the correct tumour type in almost 90% of EEC and 70% of UPSC in logistic regression. Previous studies in EEC usually evaluated single genes. Frequent methylation for *TIMP3* (Kang et al. 2006) and *CDH13* (Suehiro et al. 2008) in the present study concurs with previously published results in endometrial cancer. Methylation of the *MLH1* promoter is also a well-recognized phenomenon in (sporadic) EEC (Simpkins et al. 1999, Salvesen et al. 2000a, Zigelboim et al. 2007a,b, Esteller 2008). In consensus with our study, *MLH1* was shown to be methylated in 20–30% of cases in previous studies (Salvesen et al. 2000b, Zigelboim et al. 2007a). Methylation of *MLH1* and subsequent lack of mRNA expression are thought to be an epigenetic silencing mechanism associated with MSI in sporadic endometrial cancers. MSI is known to be present in about 25% of endometrial cancers and has been found exclusively in EEC (Risinger et al. 1993, Levine et al. 1998). As expected, we showed that *MLH1* was never methylated in UPSC.

Only a few studies have included, often small numbers, of UPSC. Therefore, previously published data have to be interpreted with caution. Risinger et al. (2003) found no promoter methylation in UPSC at all. For *MLH1*, *DAPK*, *BRCA1* and *p16*, this is in line with our results. However, in our study, *RASSF1* was frequently methylated in both EEC and UPSC. Moreover, we suggest that methylation of *TP73* and *CDKN2B* (*p15*) is relatively frequently observed in UPSC as compared to EEC (20 vs 4% and 27 vs 9%). Evaluation of *TP73* for epigenetic changes has shown aberrant promoter methylation in ~20% of UPSC. The *p53*-related *p73* gene shares functional similarities with *p53*, including the ability to transactivate *p53*-regulated genes, inhibit cell growth and induce apoptosis (reviewed by Stiewe & Putzer (2002)). For this reason, it has been proposed that *p73* is altered more frequently in cases without *p53* mutations. However, in UPSC, which often contain *p53* mutations, we found no correlation between *p73* methylation and *p53* staining. This might indicate that dysfunction of *p53* and *p73* is not exclusive. Since the transcription factor E2F1 can induce *p73* protein expression in a *p53*-deficient background (Irwin et al. 2000), silencing of *p73* could be an important mechanism for escape from *p53*-independent apoptosis. In different types of tumours, *p73* methylation is associated with aggressive behaviour, bad response to conventional chemotherapy and a worse prognosis,

suggesting a relationship between *p73* inactivation and aggressiveness of tumours (Kuo et al. 2009).

We found *RASSF1* and *APC* to be frequently methylated in both EEC and UPSC without significant preference for either tumour type. As in our present study, *RASSF1* was shown to be frequently methylated in EEC (Arafa et al. 2008, Pallares et al. 2008). However, where we found frequent methylation for *RASSF1* in UPSC in line with Liao et al. (2008) and Pallares et al. (2008) found less *RASSF1* methylation in UPSC (20%) than in EEC (61.5%). The high frequency of *APC* methylation is in line with published data, in which the methylation level reported ranged between 22 and 46% (Moreno-Bueno et al. 2002, Pijnenborg et al. 2004, Banno et al. 2006). As in our study, Ignatov et al. (2009) recently showed no significant difference between the percentage of EEC (59%) and UPSC (47%) cases methylated for *APC*.

The set of genes that we used in the present study was previously studied by Joensuu et al. (2008) in hereditary colorectal and endometrial cancers. The frequently methylated genes in our sporadic EEC correspond to those found in hereditary endometrial cancer, except for *CDKN2A* and *MLH1*. *CDKN2A* (encoding *p14^{INK}*) was found to be frequently methylated in the hereditary cases (Joensuu et al. 2008), whereas *MLH1* was frequently methylated in our sporadic cases. This suggests that sporadic and hereditary EEC may differ in methylation patterns.

Since mixed histological patterns do occur and diagnosis of UPSC on morphological features can be difficult, the histological classification used as a reference may be disputable. In some cases, there was discordance between pathologists regarding histological classification. These mixed- and disputed cases could obscure a more distinct subtype-specific methylation pattern. Hence, this tumour type-specific methylation pattern seemed not sufficiently differentiating to be of clinical use above histological features and *p53* immunohistochemical staining. A more differentiating tumour type-specific methylation signature for diagnostic purposes needs further studies and validation. Prominent was the clustering of the grade 3 tumours, irrespective of their histological classification. This may point to, in part, common epigenetic events in high grade endometrial carcinoma irrespective of their EEC or UPSC origin. Therefore, a methylation assay might be of less added value in distinguishing grade 3 EEC and UPSC.

Contradictory results have been described as to the prognostic value of *MLH1* methylation in endometrial carcinoma. In our study, *MLH1* methylation was significantly associated with a shorter DFS and OS in

EEC patients. In a multivariate model together with stage, grade and depth of myometrial invasion, *MLH1* methylation emerged as an additional prognostic factor to stage for DFS. At variance with our results, survival tended to be better for patients with methylated *MLH1* tumours in a study by Salvesen *et al.* (2000b), although statistical significance was not reached in that particular study. Others did not find a trend of *MLH1* methylation status for survival at all (Zigheboim *et al.* 2007a,b), neither *MLH1* methylation seemed to be predictive for the development of recurrences in stage I endometrial carcinoma (Pijnenborg *et al.* 2004). Where we only included EEC cases in survival analysis, Salvesen *et al.* (2000b) evaluated both EEC and UPSC in their survival analyses. The underlying heterogeneity of patient populations might therefore explain some of the discrepancies described here. The methods used to assess *MLH1* methylation could also partly explain the variability of results. Often different CpG islands within the same promoter are studied by different methods. Moreover, Varley *et al.* (2009) recently showed an unexpected heterogeneous pattern of *MLH1* promoter methylation within individual endometrial carcinomas.

In the present study, patients of all stages were included. In a subgroup analysis of stage I and II EEC patients, *MLH1* methylation retained its prognostic significance, while significance was lost in stage III/IV patients, indicating that *MLH1* methylation seems to be especially prognostically important in low stage patients. This is congruent with the observation of *MLH1* methylation being present in 7–80% of the atypical endometrial hyperplasias, suggesting that *MLH1* methylation is an early event in the tumorigenesis of sporadic EEC (Esteller *et al.* 1999, Horowitz *et al.* 2002).

In conclusion, promoter methylation for common TSGs is more frequent in EEC than in UPSC. Methylation of *CDH13*, *MLH1* and *TIMP3* seems to be characteristic for EEC, and methylation of *CDKN2B* and *TP73* seems to be characteristic for UPSC. Methylation of *CDH13* and *TP73* predicted the correct tumour type in almost 90% of EEC and 70% of UPSC, which is promising as a diagnostic test but requires further validation. Furthermore, high methylation, and especially methylation of *MLH1*, may have prognostic value in EEC.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/ERC-10-0014>.

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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