

High frequency of *RASSF1A* and *RARb2* gene promoter methylation in morphologically normal endometrium adjacent to endometrioid adenocarcinoma

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Date of submission 27 February 2008
Accepted for publication 7 May 2008

Arafa M, Kridelka F, Mathias V, Vanbellinghen J-F, Renard I, Foidart J-M, Boniver J & Delvenne P (2008) *Histopathology* 53, 525–532

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Aims: To identify a DNA methylation signature of endometrioid carcinoma of the endometrium (EEC) in the early stages of endometrial carcinogenesis.

Methods and results: Archival biopsy specimens of 39 EECs, 14 cases of atypical hyperplasia (AH), 11 histologically normal endometrial tissues adjacent to EECs and 24 normal control endometrial samples were retrieved. The cases were tested by quantitative methylation-specific polymerase chain reaction with primers hybridizing in the promoter regions of five genes frequently methylated in human cancer (*RASSF1A*, *RARb2*, *P16*, *MGMT* and *GSTPi*). Twenty-nine of 39 (74%) EECs and 7/14 (50%) AHs were methylated for the *RASSF1A* gene, whereas 17/39 (44%) EECs and

6/14 (43%) AHs were positive for the methylation of the *RARb2* gene. No significant results were obtained for the other genes (*P16*, *MGMT* and *GSTPi*). Interestingly, 4/11 (36%) and 6/11 (55%) histologically normal endometrial tissues adjacent to EEC showed, respectively, *RASSF1A* and *RARb2* gene methylation. Furthermore, these 11 specimens were microsatellite stable and showed similar proliferative, cell cycle and apoptotic mean labelling indices as the normal endometrial control tissues.

Conclusions: Promoter region methylation of *RASSF1A* and *RARb2* genes is an early event in endometrial carcinogenesis.

Keywords: carcinoma, DNA methylation, endometrium, *RARb2*, *RASSF1A*

Abbreviations: AH, atypical hyperplasia; CpG, cytosine preceding directly a guanosine base; EEC, endometrioid carcinoma of the endometrium; *GSTPi*, Pi-class glutathione S-transferase; LI, labelling index; *MGMT*, O6-methylguanine DNA methyltransferase; MSH, microsatellite instability high; MSI, microsatellite instability; MSS, microsatellite stable; PCR, polymerase chain reaction; *RASSF1A*, Ras-association domain gene family 1A; *RARb2*, retinoic acid receptors beta 2; TMA, tissue microarray; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling

Introduction

Endometrioid carcinoma of the endometrium (EEC) accounts for about 80% of cases of endometrial carcinoma, which is the most common gynaecological

malignancy in developed countries.^{1,2} Like most epithelial malignancies, EEC results from the accumulation of several abnormalities in oncogenes, tumour suppressor genes and genes involved in DNA repair. Once the tumour has developed, several additional molecular alterations occur in different neoplastic subclones. These new alterations are responsible for tumour heterogeneity, tumour invasion and metastasis.³ It has been acknowledged for more than 50 years

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that prolonged exposure to oestrogens in the absence of sufficient levels of progestogens promotes the development of atypical endometrial hyperplasia (AH) and, subsequently, of EEC. AH is usually associated with architecturally complex endometrial hyperplasia and with an increased risk of evolution to EEC (25–40%). This is in contrast to simple and complex hyperplasia without atypia, which have a much lower risk of malignant progression (1% and 3%, respectively).^{1,4} Typical risk factors for EEC are obesity, anovulatory states, early menarche, late menopause, nulliparity and unopposed exogenous oestrogens. These observations have led to the development of an integrated model of endometrial carcinogenesis involving both genetic and hormonal mechanisms.²

In recent years, it has become clear that epigenetic alterations are of importance in the regulation of gene expression in diverse disease states, including neoplasia.⁵ DNA methylation is an epigenetic phenomenon through which S-adenosyl-methionine (methyl donor) adds a methyl group to the cytosine ring. This reaction is catalysed by DNA methyl transferases and occurs in the mammalian genome in nucleotide doublets, where cytosine directly precedes a guanosine base (CpG dinucleotide). Approximately half of the human genome has CpG islands on housekeeping genes and genes with tissue-specific patterns of expression.^{6,7} DNA methylation can modify the activity of these genes without changing their sequences. Abnormal patterns of DNA methylation have been recognized in various cancers. In particular, increases in DNA methylation in gene promoter regions are associated with silencing of tumour suppressor genes and often precede apparent malignant changes, suggesting that DNA methylation assessment could be used for the early diagnosis of cancer.^{5,7–10}

DNA methylation status can be rapidly assessed by methylation-specific polymerase chain reaction (PCR). This method is based on the initial modification of DNA by sodium bisulphite, converting all the unmethylated, but not the methylated, cytosine to uracil and subsequent amplification with primers specific for methylated vs. unmethylated DNA. Moreover, this technique facilitates the detection of a low number of methylated alleles and is feasible on paraffin-embedded material.¹¹

Among the genes suspected of inducing human cancers as a consequence of hypermethylation of their promoter regions are *RASSF1A*, *RARb2*, *P16*, *MGMT* and *GSTPi*. Alterations of these tumour suppressor genes are frequently listed in various types of neoplasms.⁵ Ras-association domain gene family 1A (*RASSF1A*) is a gene located at 3p21.3 region and

is known to induce cell cycle arrest through the Rb-mediated checkpoint by inhibiting the accumulation of cyclin D1.^{12,13} Retinoic acid receptor beta 2 (*RARb2*), present on chromosome 3p, has been found to be silenced by methylation of the gene's promoter regions in a variety of human cancers, leading to increased cellular proliferation, decreased differentiation and apoptosis.^{14,15} *P16^{INK4A}* is an inhibitor of the cyclin D-dependent protein kinase 4/6, and its inactivation leads to phosphorylation of retinoblastoma protein and acceleration of the cell cycle.¹⁶ O6-Methylguanine DNA methyltransferase (*MGMT*) is a DNA repair protein that removes mutagenic adducts from the O6-guanine in DNA and hence protects against mutations.¹⁷ Pi-class glutathione S-transferase (*GSTPi*) acts as a tumour suppressor gene, as it codes for a phase II metabolic enzyme that detoxifies reactive electrophilic intermediates.¹⁸

The purpose of this study was to search for a methylation signature of EEC in the early stages of endometrial carcinogenesis.

Materials and methods

SAMPLE SELECTION

Paraffin-embedded tissue blocks of hysterectomy specimens representing 39 EECs, 14 AHs and 11 of histologically normal endometrium adjacent to EECs were retrieved from patients aged 45–88 years. Also, 24 control endometrial tissue specimens from hysterectomy specimens performed in women >50 years old for benign non-endometrial pathology were taken. The samples were obtained from the archives of the Pathology Department of the University Hospital of Liège. The protocol was approved by the Ethics Committee of the University Hospital of Liège.

DNA EXTRACTION

Before DNA extraction from the paraffin-embedded tissue blocks, a 5 µm thick section was cut from each tissue block and stained with haematoxylin and eosin to confirm the histological diagnosis and to define the purity of the normal or pathological tissue in the blocks. DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

QUANTITATIVE METHYLATION-SPECIFIC PCR

Sodium bisulphite treatment of genomic DNA was done as described previously.¹¹ Primers for detecting the presence of methylated *RASSF1A*, *RARb2*, *P16*, *MGMT*

Table 1. Sequences of primers used for the detection of methylated alleles in quantitative methylation-specific polymerase chain reaction

RASSF1A	5'-GCGTTGAAGTCGGGGTTC-3' (sense) 5'-CCCGTACTTCGCTAACTTTAAACG-3' (antisense)
RARb2	5'-TCGAGAACGCGAGCGATTTCG-3' (sense) 5'-GACCAATCCAACCGAAACGA-3' (antisense)
p16	5'-TTATTAGAGGGTGGGGCGGATCGC-3' (sense) 5'-GACCCCGAACCGCGACCGTAA-3' (antisense)
MGMT	5'-TTTCGACGTTGTAGTTTTTCGC-3' (sense) 5'-GCACTCTTCCGAAAACGAAACG-3' (antisense)
GSTPi	5'-TTCGGGGTGTAGCGGTCGTC-3' (sense) 5'-GCCCAATACTAAATCACGACG-3' (antisense)
β -actin	5'-TAGGGAGTATATAGGTTGGGGAAGTT-3' (sense) 5'-AACACACAATAACAAACACAATTCAC-3' (antisense)

and *GSTPi* genes, as well as the β -actin gene (chosen as an internal control) are shown in Table 1.

Thermal cycling was performed by iCycler (BioRad, Hercules, CA, USA) and initiated with an incubation step of 5 min at 95°C, followed by 47 cycles (95°C for 30 s, 57°C for 30 s, 72°C for 30 s). The last step was performed at 72°C for 5 min. These conditions were similar for *RASSF1A*, *RARb2*, *P16*, *GSTPi* and β -actin genes, but different for *MGMT* gene (47 cycles; 95°C for 30 s, 55°C for 30 s, 72°C for 30 s). The methylation value was the ratio between the copy number of the gene of interest/ β -actin copy number \times 1000. The highest methylation value of the control endometrial specimens from the non-cancerous patients was taken as a cut-off, above which the cases were considered to be hypermethylated.¹⁹

TISSUE MICROARRAY

The areas used for the construction of the tissue microarrays (TMAs) were selected on the slides and on the donor blocks and were sampled using a manual arraying instrument (Beecher Instruments, Sun Prairie, WI, USA). Two TMA blocks were constructed using 1-mm tissue cores (Alphelys, Plaisir, France). A total of 88 spots represented the whole series (39 EECs, 14 AHs, 11 normal endometrial tissues adjacent to EEC and 24 controls). The blocks were cut into 5- μ m sections and coated with paraffin for future use.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was carried out on the slides obtained from the TMAs with anti-Ki67 (Dako, Glostrup, Denmark) and anti-cyclin D1 (Novocastra, Newcastle, UK) antibodies. The staining procedures were carried out according to the manufacturers' instructions. The labelling index (LI) was calculated as the percentage of immunopositive nuclei of endometrial epithelial or tumour cells in 200–500 similar cells. The mean index was then calculated for each of the two markers.

APOPTOSIS ASSAY

The terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) assay for apoptosis was performed using the In Situ Cell Death Detection Kit, Alkaline Phosphatase (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. The apoptotic cells and bodies were counted in all of the endometrial epithelial or tumour cell populations in each tissue core and the LI was calculated in the same way as for immunohistochemistry.

MICROSATELLITE INSTABILITY

Five EECs, four AHs, five controls and the 11 normal endometrial tissues adjacent to EEC as well as 11 other normal paraffin blocks from the same patients (ovary, cervix, appendix or tumour-free lymph nodes) were further sectioned to assess microsatellite instability (MSI). DNA extraction was done with the MagneSil[®] Genomic, Fixed Tissue System kit (Promega, Madison, WI, USA). PCR was carried out to examine five mononucleotide repeat markers (NR-21, BAT-26, BAT-25, NR-24 and MONO-27) using the MSI Analysis System, Version 1.1 (Promega). Fluorescence PCR sizing was performed on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For each tested sample, the profile pattern of each repeat marker was compared with its equivalent in normal tissue DNA. A shift in allele size in the sample compared with a matched normal sample was scored as MSI+ for this marker.²⁰

STATISTICAL ANALYSIS

The methylation frequencies were analysed with the two-tailed *P*-values calculated using Fisher's exact test (<http://www.graphpad.com/quickcalcs/contingency1.cfm>). Differences in immunohistochemical mean LIs were determined with unpaired *t*-test (<http://www.graphpad.com/quickcalcs/ttest1.cfm>). *P*-values <0.05 were considered to be significant.

Results

QUANTITATIVE METHYLATION-SPECIFIC PCR

After an initial run of five cases from each of the diagnostic categories, valuable results were obtained for two out of the five examined genes (*RASSF1A* and *RARb2*). The different methylation values for each diagnostic category are shown in Figure 1. Considering the highest methylation value among the controls as cut-off, EEC showed *RASSF1A* gene methylation in 29/39 (74%), whereas *RARb2* gene methylation was present in 17/39 (44%) of cases (Table 2). For the different grades of EEC, *RASSF1A* and *RARb2* gene methylation was found, respectively, in 80 and 40% of grade I, 67 and 33% of grade II and 75 and 58% of grade III neoplasms. AH lesions showed *RASSF1A* and *RARb2* gene methylation in 7/14 (50%) and 6/14 (43%), respectively (Table 2). Of the 11 morphologically normal endometrial tissues adjacent to EEC, 4/11 (36%) showed methylation of the *RASSF1A* promoter region and 6/11 (55%) *RARb2* gene methylation (Table 2). In each group of lesions, some were positive for both markers and others for only one marker. The combined sensitivities of the test in detecting one or both genes together was higher in EECs and AHs, but not in the normal endometrial tissues adjacent to EEC (Table 2).

TISSUE MICROARRAY

The percentages of Ki67+ cells were very low in normal endometrial tissues adjacent to EEC (1%), in contrast to EEC (36%) and AH (13%). Similarly, the mean LIs for cyclin D1 were <1% for normal endometrial tissues adjacent to EEC compared with 14 and 8% for EEC and AH, respectively. The differences in the mean LIs between EEC and AH were significant only for Ki67 ($P = 0.0074$). As regards the apoptosis assay, the means of LIs were low in all categories (<1%). For the normal endometrial specimens (adjacent to EEC or control), higher numbers of apoptotic cells and bodies were observed in the specimens showing secretory phase changes than in those with proliferative or atrophic histology (data not shown).

MICROSATELLITE INSTABILITY

The five control samples, all 11 normal endometrial tissues adjacent to EEC and the four AHs were microsatellite stable (MSS). Two of the five tested EECs (40%) showed high MSI (MSH) as assessed by the detection of changes in repeat number in more than

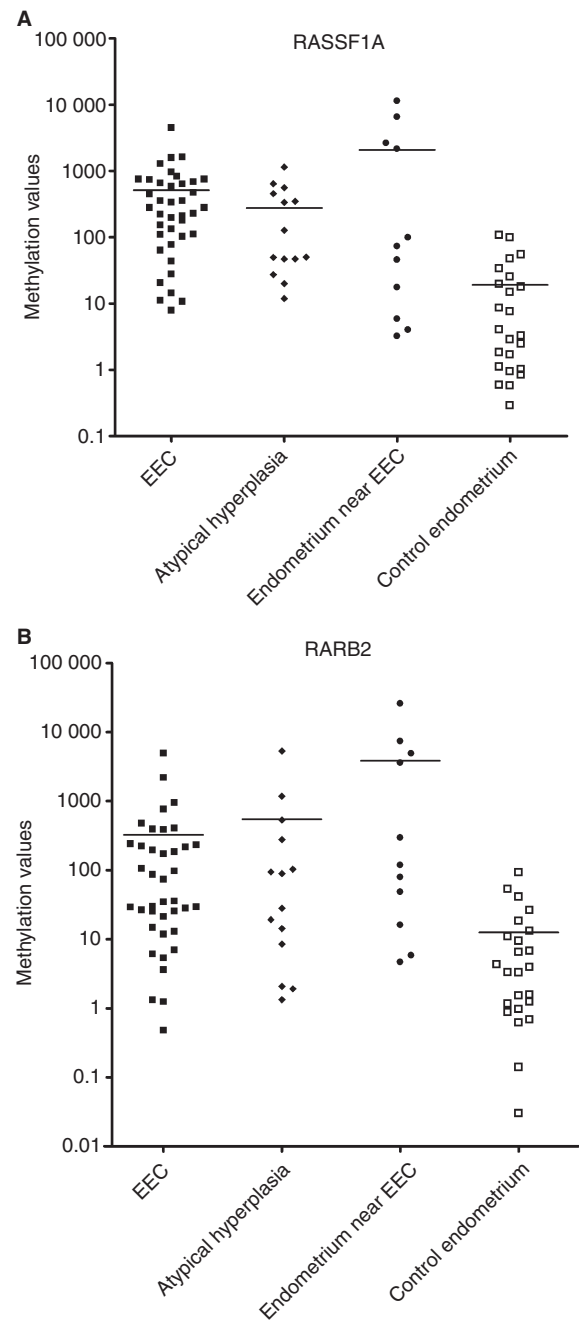


Figure 1. Scatterplot showing the methylation values in endometrioid carcinoma of the endometrium (EEC) (rectangles), atypical hyperplasia (diamonds), normal endometrial tissue adjacent to EEC (circles) and control endometrial specimens (unshaded rectangles) for *RASSF1A* (A) and *RARb2* genes (B). The horizontal lines represent the mean values. The cut-off was determined by the highest value of the control specimens from the non-cancerous patients.

one of the examined repeat markers. Representative examples of MSH and MSS in EEC and adjacent normal endometrial tissues are shown in Figures 2 and 3.

Table 2. RASSF1A and RARb2 methylation frequencies in endometrioid carcinoma of the endometrium (EEC), atypical endometrial hyperplasia (AH) and normal endometrial tissue adjacent to EEC

	RASSF1A methylation (%)	RARb2 methylation (%)	RASSF1A and/or RARb2 methylation (%)
Endometrioid carcinoma of the endometrium	74	44	77
Atypical hyperplasia	50	43	64
Endometrium adjacent to endometrioid carcinoma	36	55	55

Discussion

As endometrial carcinogenesis is a multistep process involving precursor lesions, the present study was conducted to determine whether changes in methylation can be used for the early diagnosis of EEC. Like other investigators,^{21,22} we found *RASSF1A* and *RARb2* gene methylation in a significant percentage of EECs. Interestingly, in our study normal endometrial tissues adjacent to EEC were also shown to have frequent methylation in the *RASSF1A* and *RARb2* gene promoter region (36 and 55%, respectively), suggesting the presence of multifocal epigenetic alterations during endometrial carcinogenesis. The widely variable values of CpG island methylation in this study suggest that promoter region methylation is a markedly heterogeneous process. It is believed that the methylation state is not always maintained and its extent at any locus can change dramatically from one cell to another and even from one CpG site to another.^{23,24} To explore further the effect of epigenetic silencing of these two genes, we examined proliferative activity (Ki67) and cell cycle progression (cyclin D1) by immunohistochemistry. It is known that *RASSF1A* gene induces cell cycle arrest by inhibiting the accumulation of cyclin D1. Furthermore, the induction of apoptosis may be one of the effects of *RASSF1A* as a tumour suppressor gene.²⁵ Similarly, epigenetic silencing of *RARb2* gene expression may be sufficient to enhance malignant transformation by modifying proliferation, differentiation and apoptosis. This could occur without the need for the mutated *p53* gene.¹⁵ The lack of significant increases in Ki67 and cyclin D1 protein expression in normal endometrial tissues adjacent to EEC compared with the controls may be related to the efficiency of other unaffected mechanisms guarding against carcinogenesis. However, there were similar stepwise increases of the mean immunohistochemical IIs (Ki67 and cyclin D1) and methylation frequencies (*RASSF1A* and *RARb2*) throughout the shift from normal endometrium to AH and from AH to EEC. These findings support the hypothesis that genetic alterations

accumulate along the transformation process ranging from normal to premalignant and invasive patterns. The TUNEL assay also failed to demonstrate a correlation between methylation status and apoptotic indices in the 11 normal endometrial tissues adjacent to EEC. In addition, these tissues were also MSS, indicating that *RASSF1A* and *RARb2* gene methylation might precede MSI in the initial stages of endometrial carcinogenesis.

Tumour suppressor gene methylation changes in the normal endometrium adjacent to EEC, even in the absence of morphological or other molecular alterations, might reflect a field cancerization process. This hypothesis, proposed more than half of a century ago, predicts that multiple cells form independent epithelial tumours because carcinogenic exposure affects multiple cells in the field. Another primary or synchronous tumour may therefore arise from independent genetic events. This concept originated from the observation that a significant percentage of individuals with oral cancer had multiple upper aerodigestive tract tumours and from the finding of multiple invasive foci associated with overlying areas of *in situ* squamous carcinoma.²⁶ Although this hypothesis was first described for tumours strongly related to carcinogenic factors such as cigarette smoking, the view expanded over the next five decades to cover tumours of other organs including the female genital tract.^{27,28} Much attention has been paid in recent years to the aberrant molecular (pre) neoplastic changes occurring in the grossly normal appearing tissue surrounding cancer. Although there is a large body of literature reporting methylation frequency in solid cancers, few studies have analysed DNA methylation in normal and preneoplastic tissues adjacent to tumours.^{8-10,12,15,29,30}

Kanaya *et al.* observed frequent hypermethylation of the *MLH-1* gene promoter in normal endometrium adjacent to endometrial carcinoma, supporting the notion that hypermethylation of mismatch repair genes is the initial step that triggers various genetic events, namely MSI, in EEC carcinogenesis.³¹ As we have demonstrated the presence of early *RASSF1A* and

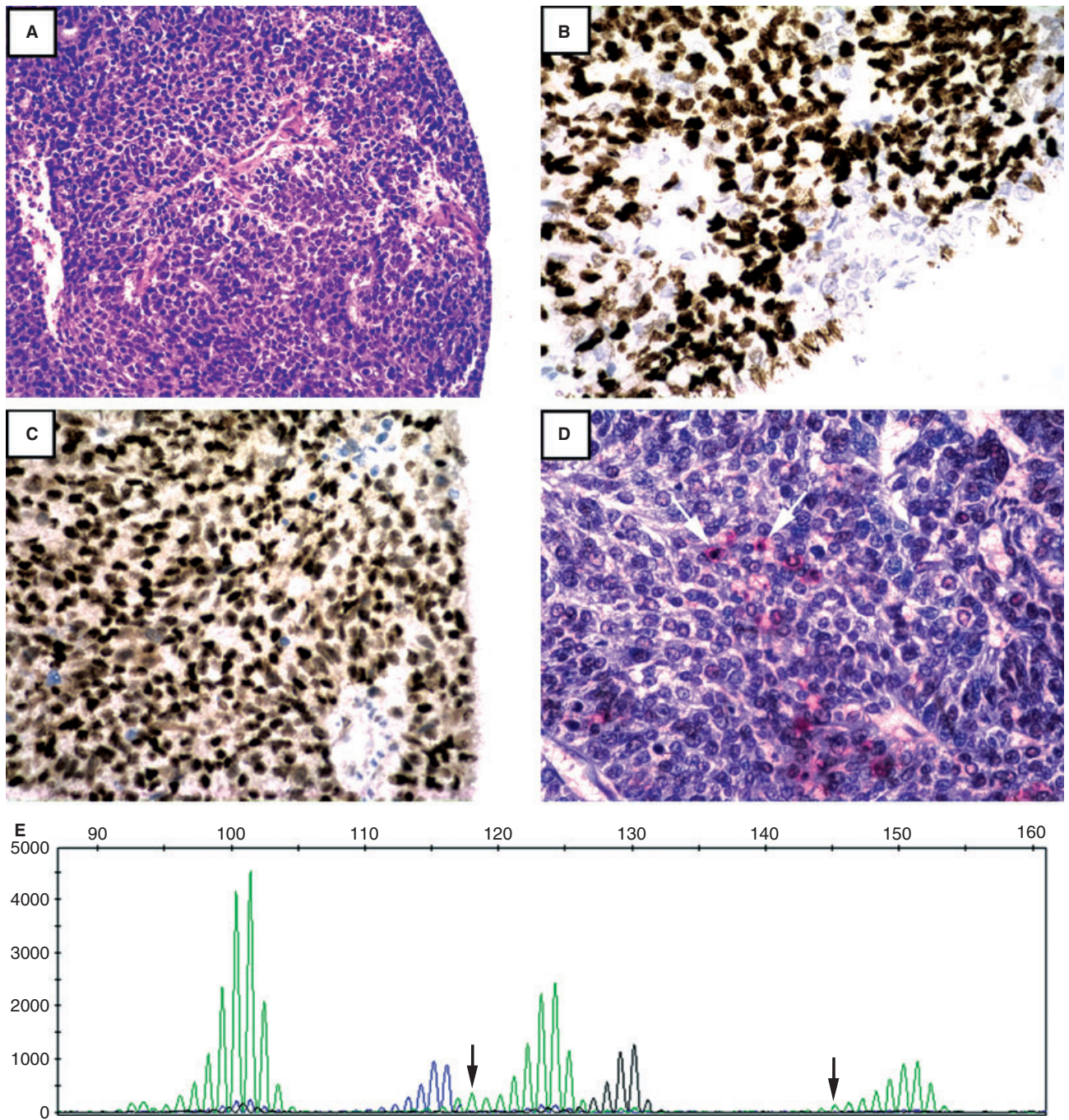


Figure 2. Representative example of endometrioid carcinoma of the endometrium with methylation of *RASSF1A* and *RARb2* genes (H&E) (A), marked proliferative activity (Ki67 immunoperoxidase) (B), high cyclin D1 labelling index (immunoperoxidase) (C), scattered deeply eosinophilic apoptotic cells and bodies (arrows) (TUNEL assay) (D) and high microsatellite instability pattern (arrows) (E).

RARb2 gene methylation in morphologically normal endometrial cells without the appearance of MSI, our results suggest that the DNA methylation process starts in these two genes before it expands to involve the *MLH-1* gene promoter and subsequent evolution to MSI.

In this study, the possible role of aberrant methylation in the progression of the disease has been demonstrated by the higher methylation frequencies with increasing severity of the lesion. On the other hand, and more importantly, the occurrence of specific methylation patterns in normal endometrial cells

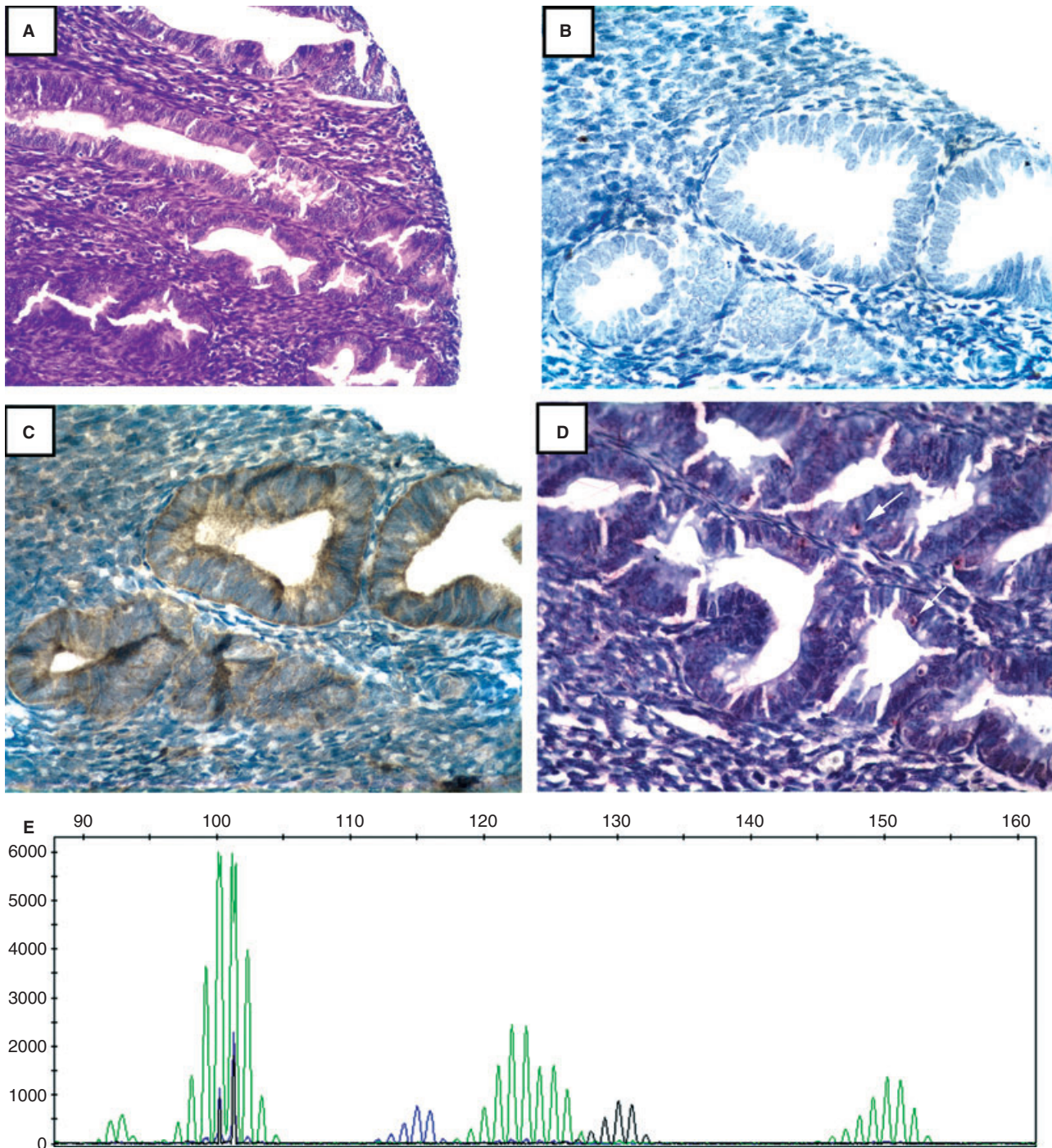


Figure 3. Representative example of normal endometrium adjacent to endometrioid carcinoma of the endometrium with methylation of *RASSF1A* and *RARb2* genes (H&E) (A), with no proliferative activity (Ki67 immunoperoxidase) (B), cyclin D1 immunonegativity (immunoperoxidase) (C), deeply scattered eosinophilic apoptotic cells and bodies (arrows) (TUNEL assay) (D) and microsatellite stable pattern (E).

adjacent to EEC favours the role of methylation imbalance in tumour initiation.^{23,32} Even if it is not yet clear whether aberrant DNA methylation is a result, a cause or both, of cancer development, our

study has demonstrated that promoter region methylation of *RASSF1A* and *RARb2* genes may be among the earliest events of endometrial carcinogenesis, as this epigenetic event occurs in morphologically normal

endometrial glandular cells before the appearance of any discernible histopathological changes. These data suggest that DNA methylation assessment may be used for the early diagnosis of EEC. In addition, as DNA methylation is a reversible process, novel demethylating agents could help to treat or to prevent EEC.

Acknowledgements

The authors thank Oncomethylome Sciences (OMS) and Patrick Roncarati for technical support. This work was supported by the Belgian Fund for Medical Scientific Research, the L. Fredericq Fund, the Centre Anti-Cancéreux près l'Université de Liège, the Marshall Programme of the Walloon Region (Neoangio no. 616476) and the Egyptian Ministry of Higher Education. P.D. is a senior Research Associate of the Belgian National Fund for Scientific Research (FNRS).

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