Epigenetic aberration is known to be important in human carcinogenesis. Promoter methylation status of RAS effector related genes, RASSF1A, RASSF2A, hDAB2IP (m2a and m2b regions) and BLU, was evaluated in 76 endometrial carcinomas and their non-neoplastic endometrial tissue by methylation specific PCR. Hypermethylation of at least one of the 5 genes was detected in 73.7% of carcinomas. There were significant correlations between methylation of hDAB2IP and RASSF1A, RASSF2A (p = 0.042, p = 0.012, respectively). Significantly, more frequent RASSF1A hypermethylation was found in Type I endometrioid carcinomas than Type II carcinomas (p = 0.049). Among endometrial cancers, significant association between RASSF1A hypermethylation and advanced stage, as well as between methylation of hDAB2IP at m2a region with deep myometrial invasion (p < 0.05) was observed. mRNA expression of RASSF1A, RASSF2A and BLU in endometrial cancer cell lines significantly increased after treatment with the demethylating agent 5-Aza-2'-deoxycytidine supporting the repressive effect of hypermethylation on their transcription. Immunohistochemical study of DNMT1 on eight normal endometrium, 16 hyperplasia, and 79 endometrial carcinomas showed progressive increase in DNMT1 immunoreactivity from normal endometrium to endometrial hyperplasia and endometrial carcinomas (p = 0.001). Among carcinomas, distinctly higher DNMT1 expression was observed in Type I endometrioid carcinomas (p < 0.001). DNMT1 immunoreactivity correlated with RASSF1A and RASSF2A methylation (p < 0.05). The data suggested that hypermethylation of RAS related genes, particularly RASSF1A, was involved in endometrial carcinogenesis with possible divergent patterns in different histological types. DNMT1 protein overexpression might contribute to such aberrant DNA hypermethylation of specific tumor suppressor genes in endometrial cancers.

Key words: DNA hypermethylation; endometrial cancer; DNMT1; RASSF1A; RASSF2A; hDAB2IP; BLU

Endometrial cancer is the one of the most common cancers in female genital tract worldwide. It can be classified into two major subtypes with respect to histopathology, cell biology and clinical course. Approximately 70–80% endometrial cancers were designated as Type I carcinomas, which show an endometrioid differentiation. They are associated with unopposed estrogen exposure and are often preceded by premalignant endometrial hyperplasia, with an overall favorable outcome. Common genetic changes include mutations of K-RAS and PTEN gene, microsatellite instability (MSI) and alteration of β-catenin. Type II carcinomas, mainly composed by serous and clear cell carcinomas, follow estrogen unrelated pathway. They often arise in the background of atrophic endometrium and are characterized by an aggressive clinical course and poor prognosis. p53 mutation and E-cadherin alteration are frequently observed in Type II cancers.

Promoter hypermethylation is a common epigenetic mechanism for tumor suppressor gene inactivation in human cancer and a promising target for molecular detection. The methylation of mammalian genomic DNA is catalyzed by DNA methyltransferases (DNMTs) family which can be divided into maintenance and de novo methyltransferases. Maintenance DNMT1 is the most abundant methyltransferase in mammalian cells. Increased expression of DNMT1 mRNA and protein has been documented in various cancers in association with poor differentiation and unfavorable outcome.

RAS proteins are key signal transducers in various important pathways. By activating downstream effectors, RAS regulates cell functions including cell proliferation, cell survival and cell morphology. K-RAS mutation was observed in about one third of Type I endometrial cancers. The RAS-association domain family (RASSF) is a new family of negative effectors of RAS protein. RASSF association domain family 1, isoform A (RASSF1A) at 3p21.3 and RAS association domain family 2, isoform A (RASSF2A) located at 20p13 are two members of this family that function as tumor suppressor genes. They directly bind to RAS in a GTP-dependent fashion via the RAS effector domain. Activated RAS-GTP can enhance RASSF1 and RASSF2 induced apoptosis and cell cycle arrest. The human DAB2 interactive protein gene (hDAB2IP) located on chromosome 9q33.1-q33.3 is a novel member of the RAS GTPase-activating gene family and seems to be a tumor suppressor. It has a negative regulatory effect on the RAS-mediated signal pathway and tumor necrosis factor-mediated apoptosis.

In this study, the methylation status of this panel of RAS effector related genes, RASSF1A, RASSF2A and hDAB2IP was investigated in endometrial cancers and correlated with clinicopathological parameters. BLU gene, an E2F regulated stress-responsive gene, and a candidate tumor suppressor gene at 3p21.3 immediately upstream of RASSF1A was also studied. The effects of demethylating agents on the expression of these genes were further explored. Immunohistochemical study of DNMT1 expression in endometrial cancers and precancerous lesions was investigated and correlated with the methylation status of these RAS-related genes.

Material and methods

Clinical samples

All specimens of endometrial tissues were collected at the Department of Pathology, the University of Hong Kong, Queen Mary Hospital. Formalin fixed, paraffin embedded tissues of 76 cases of endometrial cancers and in 15 of the 76 cases being investigated, their corresponding non-neoplastic endometrium, was also retrieved for methylation study. Prior to DNA extraction, hematoxylin and eosin-stained section was reviewed to confirm histological diagnosis and purity of the sample. Only samples with more than 75% composed of cancer were used in experiments. Among the 76 endometrial cancers included for methylation studies, there were 65 cases of endometrioid carcinomas, five cases of...
serous carcinomas and six cases of clear cell carcinomas. The average age of these patients was 57.9 years (range 34–97 years). There were 62 early-stage (Stage I) and 12 late-stage (Stage II-IV) cases, whereas two cases had no staging data. Seventy-nine cases of endometrial cancers were retrieved for DNMT1 immunohistochemistry, including 63 endometrioid carcinomas, 6 serous carcinomas and 10 clear cell carcinomas. Among these, 63 cases were also included in 76 cases used for methylation studies. Eight samples of normal endometrium (including 4 proliferative, 3 secretory and 1 atrophic endometrium), 16 cases of hyperplastic endometrium without atypia (including 7 simple hyperplasia and 9 complex hyperplasia) and 40 cases of atypical complex hyperplasia were also selected for study. Among the 40 atypical complex hyperplasia, 24 cases subsequently developed endometrioid carcinomas.

DNA extraction and bisulfite modification

Ten consecutive 10 µm-thick sections from each tissue block were deparaffinized and genomic DNA was extracted using the conventional phenol/chloroform method following the proteinase K digestion. For bisulfite treatment, 1 µg of genomic DNA was denatured by NaOH and modified by sodium bisulfite, which converts all of the unmethylated cytosines to uracils, whereas methylated cytosines remain unchanged. The modified DNA was purified using a Wizard DNA cleanup system (Promega, Madison, WI, USA) following the manufacturer’s instructions.

Methylation-specific PCR

DNA methylation was determined by methylation specific PCR (MS-PCR). The primer sequences of RASSF1A, RASSF2A, hDAB2IP and BLU were listed in Table I. The promoter region of hDAB2IP was divided into m2a (237bp) and m2b (401bp) regions. The reaction volume of 25 µl contained 100 ng bisulfite-modified DNA, 1.5 mM MgCl₂, 0.25mM dNTP, 0.5 µl each primer and 1 U Hotstar Taq (Qiagen, Valencia, CA, USA). Touchdown PCR was conducted for amplification of hDAB2IP at m2a, m2b unmethylated alleles as well as both methylated and unmethylated alleles of BLU gene. Normal lymphocyte DNA was methylated in vitro with Sssl methyltransferase and used as positive control for methylated alleles. Genomic DNA not treated for bisulfite modification and water blanks without added DNA were included as negative controls in each assay. PCR products were analyzed on 2% agarose gel containing ethidium bromide. The PCR for all samples demonstrating methylation was repeated at least once.

Cell lines and demethylation treatment

Human endometrial cancer cell lines HEC-1A and RL95-2 (American Type Culture Collection, Manassas, USA), maintained in McCoy’s 5a medium and 1:1 mixture of Dulbecco’s modified Eagle’s medium and F-12k medium with 0.005 mg/ml insulin (Sigma chemicals co. St. Louis, USA) respectively, were treated with 0, 5 µM and 10 µM 5-aza-2’-deoxycytidine (5-aza-dC) (Sigma, St. Louis, USA) for 3 days. The medium and drugs were freshly replaced every 24 hr. TRIZOL reagent (Invitrogen, Carlsbad, USA) was directly added to the cells for RNA isolation.

Quantitative real time RT-PCR

First-strand cDNA was synthesized from 2.5 µg total RNA with oligo-dT primer and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. Quantitative real-time RT-PCR was performed in a 20 µl reaction, which included 1 µl of cDNA template, 10 pmol/l of each forward and reverse primer, and 10 µl SYBR Green Mix (Bio-rad, Hercules, USA), using the iCycler Detection System (Bio-rad, Hercules, USA). The primer sequences were listed in Table I. Each PCR reaction was optimized to ensure that a single PCR product was amplified and no product corresponding to primer-dimer pairs was present. PCR reactions of each template were performed in duplicate in one 96-well plate. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and 60°C for 1 min. The relative fold change was calculated using the relative quantification gene expression compared with β-actin.

Immunohistochemistry

Five micrometer thick sections of formalin-fixed, paraffin-embedded tissues were deparaffinized and dehydrated. Antigen retrieval was conducted by heating in a pressure cooker filled with 10 mM EDTA buffer (pH 8.0) for 7 min. Endogenous peroxidase activity was blocked by incubating sections in 3% H₂O₂. After treatment with 10% normal donkey serum for 1 hr to block any nonspecific reaction, the sections were incubated with a goat anti-human polyclonal antibody against DNMT1 (N16, dilution 1:50; Sigma chemicals co. St. Louis, USA) for 3 days. The medium and drugs were freshly replaced every 24 hr. TRIZOL reagent (Invitrogen, Carlsbad, USA) was directly added to the cells for RNA isolation.

For each sample, at least 500 cells were randomly counted. Only distinct nuclear staining was considered as positive. The incidence of DNMT1 immunoreactivity in each sample was categorized into low (<20% of the counted cells) and high (20–100%) expression groups.
Advanced stage of the carcinoma correlated with methylation of BLU in endometrial cancers (compared to Type II carcinomas (3/11, 27.2%) (40/65, 61.5%) and the frequency was significantly higher when shown in marginal association between methylation of hDAB2IP because correlations between methylation of RASSF1A and BLU at m2a and m2b regions was found in only 11 (14.5%) and less frequently methylated. While aberrant methylation of RASSF1A and BLU was more frequently observed in tumors with deep myometrial invasion (5/15, 33.3%) compared to those with no or superficial myometrial invasion (4/50, 8%) (p = 0.025). No correlation between methylation of these genes and tumor grade, cervical involvement, vascular space infiltration and prognosis was found as demonstrated in overall endometrial cancer (p > 0.05).

**RASSF1A, RASSF2A and BLU mRNA expression in endometrial cancer cell lines after treatment by 5-aza-dC**

To confirm whether promoter hypermethylation contributes to reduced expression of RASSF1A, RASSF2A and BLU in endometrial cancer cell lines, we examined the effect of 5-aza-dC that inhibits DNA methylation. After treatment with 5-aza-dC for 3 days, significant elevated mRNA expression of these genes, ranged from 4- to 2,500-folds, was observed in both HEC-1A and RL95-2 cells (Fig. 2).

**DNMT1 protein expression in normal, hyperplastic endometrium and endometrial cancers**

DNMT1 expression in these endometrial lesions was shown in Figures 3 and 4. The immunoreactivity was demonstrated in the nuclei of epithelial and stromal cells in functional layer of proliferative endometrium while secretory and atrophic endometrium showed negative expression. There was a progressive increase in DNMT1 expression level among normal endometrium, simple and complex hyperplasia of endometrium without atypia, atypical complex hyperplasia and endometrioid carcinoma (p = 0.001).

Significant differences between normal endometrium and endometrial hyperplasia without atypia (p = 0.018), between endometrial hyperplasia without atypia and endometrial carcinomas (p = 0.036), between atypical complex hyperplasia and endometrioid carcinomas (p = 0.031) were observed. However, there was no significant difference between endometrial hyperplasia with and
expression level of these genes increased significantly after drug treatment. Real-time PCR after treatment with the indicated concentration of 5-Aza-dC for three days and normalized by housekeeping gene DNMT1 expression. DNMT1 expression level was significantly higher in endometrial cancer compared to non-neoplastic tissue. The frequency of aberrant methylation was distinctly higher in Type I endometrial carcinomas (61.5%) when compared with Type II cancers (27.2%) suggesting that the two types of cancers may adopt different genetic and epigenetic pathways, and this feature may be helpful in distinguishing the two subtypes of endometrial cancers.

**Correlation between expression of DNMT1 and DNA hypermethylation**

We evaluated correlations between DNMT1 expression and DNA methylation of these RAS related gene in 63 cases of endometrial cancers. DNMT1 protein expression level was significantly correlated with hypermethylation of RASSF1A and RASSF2A (Spearman test, \( p = 0.043, p = 0.004 \), respectively) but not with the other genes \( p > 0.05 \).

**Discussion**

In the current study, most endometrial carcinomas (75%) showed methylated alleles at 1 or more of the 5 gene loci studied while such hypermethylation was absent in the corresponding non-neoplastic tissue. The frequency of aberrant methylation ranged from 56.6% at RASSF1A to 13.2% at hDAB2IP at m2b. On the other hand, significantly increased RASSF1A, RASSF2A and BLU mRNA expression was demonstrated in endometrial cancer cell lines after treatment with 5-aza-dC. Promoter hypermethylation of these genes may thus be involved in transcriptional inactivation of their expression in endometrial cancers although hypermethylation of hDAB2IP and BLU may occur at CpG islands not tested in this study. This is the first study reporting the methylation status of RASSF2A, hDAB2IP and BLU in endometrial carcinomas.

An inverse correlation was observed between K-RAS mutations and hypermethylation of RASSF1A in MSI-associated endome-trial, colorectal\(^{31}\) and pancreatic carcinomas.\(^{32}\) In endometrial cancers, RASSF1A hypermethylation has been reported to be associated with advanced stage disease,\(^{34,35}\) while association with risk of recurrent cancer and poor survival is controversial.\(^{34,35}\) Our studies confirmed that RASSF1A hypermethylation was significantly associated with advanced staging of the cancers but did not correlate with survival. In addition, the frequency of RASSF1A hypermethylation was distinctly higher in Type I endometrial carcinomas (61.5%) compared with Type II cancers (27.2%) suggesting that the two types of cancers may adopt different genetic and epigenetic pathways, and this feature may be helpful in distinguishing the two subtypes of endometrial cancers.

RASSF2A methylation is one of the most frequent epigenetic events (70–72.6%) in sporadic colorectal cancer and its frequency correlates with K-RAS/BRAF mutation. It is an early event in colorectal tumorigenesis, detectable in most of the colon adenomas.\(^{13,37}\) We observed that RASSF2A is less frequently methylated in endometrial cancers compared to RASSF1A. No association was found between RASSF2A and RASSF1A methylation. RASSF2A hypermethylation was found more frequently in samples from old patients. Similar observation was found in colorectal cancer.\(^{13}\)

Transcriptional silencing by aberrant methylation of the P2 promoter region of hDAB2IP played an important role during the tumorigenesis of various cancers.\(^{30,39,39}\) Aberrant methylation was significantly associated with the presence of lymph node metastasis in lung and breast cancers.\(^{30,38}\) While the m2a region appeared to be the key regulatory region for hDAB2IP expression in prostate cancer,\(^{37}\) downregulation of hDAB2IP expression correlated more with the methylation of m2b region in gastrointestinal tumors.\(^{39}\) Interestingly, although it seems that hDAB2IP methylation is not a frequent event in endometrial cancers (14.5% in the m2a and 13.2% in m2b region), most of the cases that showed methylation of the hDAB2IP gene displayed the same methylation status in both regions, a finding also detected in other tumors.\(^{30,38,39}\) Methylation of m2a regions was also found to be significantly correlated with deep myometrial invasion in endometrioid cancers.
BLU gene was usually studied with RASSF1A as its promoter region is within 6kb of the RASSF1A promoter. The association between methylation of BLU and RASSF1A in various cancers was conflicting. Methylation of BLU was observed in primary nasopharyngeal carcinoma (66%), glioma (80%), small cell (14%) and nonsmall cell lung cancers (19–43%), as well as neuroblastoma (41%). Nevertheless, no correlation between the hypermethylation of BLU and RASSF1A was observed in aforementioned tumors except nonsmall cell lung cancer. In our investigation, methylation of BLU was seen in 24% of endometrial cancers. Again, no association between BLU and RASSF1A methylation in endometrial cancers was observed, supporting that methylation of BLU and RASSF1A are independent events. However, methylation of BLU was found to be correlated with hDA-B2IP in Type I endometrioid carcinomas.

This is also the first study reporting DNMT1 protein expression profile by immunohistochemistry in endometrial cancers and its precursor lesions. In non-neoplastic endometrium, DNMT1 was expressed in functional layer of proliferative endometrium but not in secretory and atrophic endometrium. Similar DNA methylation profiles in different phrases of endometrium was described in an
earlier study by Ghabreau et al. using 5-methyl cytidine as methylation marker. The expression level of DNMT1 immureactivity increased in endometrial hyperplasia with or without atypia when compared to normal endometrium. An even higher expression level of DNMT1 was observed in endometrial cancers, particularly in the Type I cancers, suggesting that DNMT1 protein overexpression is a very early and progressive event in the multistep endometrial tumorigenesis. Such progressive increase in DNMT1 expression has been reported in development of uterine cervical squamous carcinoma, pancreatic and hepatocellular carcinomas.

In endometrial cancers, two distinct DNMT1 protein expression patterns were identified, being significantly higher in Type I cancers compared to Type II cancers. Our results concur with previous observation that upregulation of DNMTs mRNA was detected in endometrioid cancers while downregulation of DNMTs occurred in serous cancers. Taken together with the finding of more frequently RASSF1A hypermethylation in Type I endometrioid carcinomas, divergent DNA methylation pathways clearly exist in the development of the two types of endometrial cancers. As DNMT1 protein overexpression is a very early event in multistep endometrial carcinogenesis and is present in virtually all the endometrioid cases, it is logical that the DNMT1 protein score did not correlate further with various clinicopathological parameters reflecting tumor differentiation or progression, such as grade, stage and myometrial invasion.

Reports had shown that DNMT1 mRNA or protein overexpression significantly associated with the CpG island methylation phenotype in various cancers. In this study, we also observed a significant association between DNMT1 protein expression and RASSF1A and RASSF2A hypermethylation. DNMT1 is known to be a maintenance methyltransferase in mammalian cells. However, recent studies found that DNMT1 also accounts for the majority of de novo methyltransferase activity in protein extracts from human cancer cells. In addition, DNMT1 and DNMT3b catalyze de novo methylation cooperatively rather than independently. Our data further confirmed that DNMT1 might have more diverse catalytic activities rather than previously suspected a simple maintenance enzyme. DNMT1 may play an important role in initiating promoter CpG island hypermethylation associated with gene silencing in endometrial carcinogenesis.

In conclusion, we demonstrated hypermethylations of RAS related genes in endometrial carcinomas in association with distinct clinicopathological parameters. DNMT1 activity might contribute to aberrant DNA hypermethylation of these RAS-related genes. Increased DNMT1 protein expression is involved in the multistage endometrial tumorigenesis from hyperplastic to malignant endometrium. Two distinct RASSF1A hypermethylation and DNMT1 protein expression patterns were observed in Type I and Type II endometrial cancers, suggesting that divergent epigenetic pathway may be involved in Type I and Type II endometrial carcinogenesis. Evaluation of RASSF1A methylation status and DNMT1 protein expression may be potentially useful biomarkers to enhance diagnosis and classification of endometrial cancers. Since conservative medical therapy is sometimes administered in patients with endometrial hyperplasia or low grade cancer who want to preserve their fertility and avoid surgery, these assays may also help in monitor of patients under such treatment.

Acknowledgements

Dr. Xiaoyun Liao was partially supported by Chan WC scholarship, the Hong Kong College of Pathologists and postgraduate studenthip, the University of Hong Kong.

References


