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DNA methylation as a biomarker for the detection of hidden carcinoma in endometrial atypical hyperplasia

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HIGHLIGHTS

- *AJAP1*, *HS3ST2* and *SOX1* methylation analysis is a potential method for detection of endometrial carcinoma hidden in atypical hyperplasia.
- Testing the methylation status of candidate genes may assist in devising an adequate treatment strategy prior to major surgery.
- AJAP1, HS3ST2 and SOX1 expression may help to differentiate premalignant endometrium and endometrial carcinoma.

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ABSTRACT

Objective. Women with atypical hyperplasia (AH) are often found to have endometrial carcinoma (EC) at hysterectomy. The purpose of this study was to evaluate whether the hypermethylation of specific genes found by methylomic approaches to the study of gynecologic cancers is a biomarker for EC in women with AH.

Methods. We evaluated the methylation of *AJAP1*, *HS3ST2*, *SOX1*, and *PTGDR* from 61 AH patients undergoing hysterectomy. Endometrial biopsy samples were analyzed by bisulfite conversion and quantitative methylation-specific polymerase chain reaction. A methylation index was used to predict the presence of cancer. To confirm the silencing effects of DNA methylation, immunohistochemical analysis of AJAP1, HS3ST2, and SOX1 was performed using tissue microarray.

Results. Fourteen (23%) patients had EC at hysterectomy. *AJAP1*, *HS3ST2*, and *SOX1* were highly methylated in the EC patients' biopsy samples ($p \leq 0.023$). AJAP1, HS3ST2, and SOX1 protein expression was significantly higher in patients with AH only ($p \leq 0.038$). The predictive value of *AJAP1*, *HS3ST2*, and *SOX1* methylation for EC was 0.81, 0.72, and 0.70, respectively. Combined testing of both *AJAP1* and *HS3ST2* methylation had a positive predictive value of 56%, methylation of any one of *AJAP1*, *SOX1*, or *HS3ST2* had a 100% negative predictive value.

Conclusions. Hypermethylation of *AJAP1*, *HS3ST2*, and *SOX1* is predictive of EC in AH patients. Testing for methylation of these genes in endometrial biopsy samples may be a hysterectomy-sparing diagnostic tool. Validation of these new genes as biomarkers for AH screening in a larger population-based study is warranted.

Introduction

Endometrial carcinoma (EC) is one of the most common cancers of the female genital tract, although the incidence varies between countries [1]. Prolonged exposure to estrogen promotes the development of endometrial hyperplasia (EH), which leads to atypical hyperplasia (AH); 25–40% of patients with AH subsequently progress to EC. Although AH is the least common type of hyperplasia, it is the type

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most likely to progress to type 1 EC, which accounts for more than 80% of uterine cancers [2]. EC is usually confined to the inner lining of the uterus, which can be removed by hysterectomy. Unfortunately, even in stage I EC, there is a significant risk of tumor recurrence, distant metastasis, and death [3].

EH is classified into two categories by the World Health Organization: 1) EH, including simple and complex hyperplasia without atypia, and 2) endometrial AH, including simple and complex hyperplasia with atypia [4]. Several studies have shown that cytological atypia, which is the major criterion for the diagnosis of AH and the most reliable indicator of progression from EH to EC, has poor reproducibility [2,5,6]. In 12.7–42.6% of cases, EC coexists in patients with a diagnosis of AH [7]. The high rate of unrecognized cancer among women diagnosed preoperatively with AH reflects the fact that the histologic criteria for differentiating AH from some types of EC on dilation and curettage (D&C) are controversial and subject to different interpretations [8–10].

Because of overlap in the histologic picture of AH and low-grade EC in the limited tissue sample evaluated before major surgery, differentiation on pathologic grounds alone can be very difficult or impossible [5,10,11]. Although D&C before hysterectomy is the gold standard method for the diagnosis of endometrial lesions, detection of AH cannot rule out a more severe lesion [12,13]. EC found at the time of hysterectomy for AH may be associated with deep myometrial (10%) or cervical stroma (5%) invasion [14].

Hysterectomy is the main therapeutic modality for AH. Conservative approaches such as high-dose progestin may be acceptable treatment options in certain situations (*e.g.*, to maintain fertility), but the risks of progression to malignancy and of concurrent EC remain high [14]. At present, there is no established biomarker to differentiate endometrial AH and EC. Such a marker could be hysterectomy sparing for AH patients without EC. Even when EC arising from endometrial premalignant lesions is clearly defined, the possibilities for EC screening are very limited. Reliable determination of the presence or absence of EC would allow for better surgical decisions about hysterectomy and staging. The reassurance of patients given fertility-sparing management for AH may alleviate unnecessary anxiety. There is, therefore, a need to develop new, molecular-based, complementary tools that could improve the pathological diagnosis.

Epigenetic studies have demonstrated that silencing of genes, such as tumor-suppressor genes (TSGs), can act as a mechanism of carcinogenesis [15,16]. The addition of a methyl group to the cytosine–guanine (CpG) island results in gene silencing. Because epigenetic silencing of TSGs by promoter hypermethylation is observed commonly in human cancers, it is possible that DNA methylation could be used for the early diagnosis of cancer. This concept, and its application in gynecologic cancers, has been gaining acceptance during the past few years, especially in diagnosing and treating cervical cancer (screening and triage) and ovarian cancer (prognosis) [17–20]. However, similar studies of EC are relatively limited. It is known that the progression of EC involves a multistep process, and both genetic and epigenetic events have been shown to play important roles. Although gene promoter CpG islands epigenetically marked by *de novo* DNA methylation may serve as biomarkers in EC, they have been rarely studied in AH [21–24]. Such epigenetic biomarkers could be useful for identifying EC in AH.

Our previous research on the epigenomics of cervical cancer using methylomic approaches identified several candidate genes that are methylated in cervical cancer tissues. Several candidate genes were significantly hypermethylated in CIN3+ lesions [19,25]. Because the uterine cervix and endometrium both originate from the Müllerian duct system, this close embryologic relationship between the uterine cervix and endometrium may be reflected in adulthood in the form of malignant lesions. We hypothesized that some of the genes hypermethylated in cervical cancer may also be hypermethylated in EC. We initially tested 28 development-related genes. To test further the feasibility of using these new biomarkers in identifying endometrial lesions, we converted the methylation analysis to a quantitative methylation-specific polymerase chain reaction (QMSP) approach and tested its application value. We found that the following several genes were potentially implicated in endometrial carcinogenesis: adherens junction-associated protein 1 (*AJAP1*), heparan sulfate D-glucosamyl 3-O-sulfotransferase-2 (*HS3ST2*), sex-determining region Y, box 1 (*SOX1*), prostanoid receptor gene, prostaglandin D_2 receptor (*PTGDR*), and LIM-homeobox gene 1A (*LMX1A*). These candidate genes, which could be used for the triage of AH, were validated in The Cancer Genome Atlas (TCGA) EC database. The aim of the present study was to analyze the DNA methylation status of *AJAP1*, *HS3ST2*, *SOX1*, *PTGDR*, and *LMX1A* genes as biomarkers for EC diagnosis in patients with endometrial AH. The discovery of reliable epigenetic biomarkers for diagnosis may open a new avenue for the management of AH patients with and without EC.

Materials and methods

Patients and clinical samples

Samples of endometrium from patients with endometrioid-type EC ($n = 20$; 8 G1 cases, 8 G2 cases, 4 G3 cases) and with dysfunctional uterine bleeding ($n = 20$) were included as cancer and normal controls, respectively. Specimens were obtained from tissue blocks for methylation analysis of the candidate genes. Endometrial biopsy tissues of patients with AH ($n = 61$) were collected for methylation analysis. All patients underwent hysterectomy within 3 months after endometrial sampling. The clinicopathologic characteristics of patients were recorded by the data managers from the Gynecologic and Pathological Center at the Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan, R.O.C., who reviewed the patients' pathologic diagnosis and recorded their surgical status. The final diagnosis was made according to the worst pathologic finding from endometrial sampling or hysterectomy. Informed consent was obtained from all patients, and this study was approved by the Institutional Review Board of the Tri-Service General Hospital (TSGHIRB No: 2-101-05-041).

DNA extraction, bisulfite conversion, and QMSP

Before extraction of DNA from the paraffin-embedded tissue blocks, a 5 μm-thick section was cut from each tissue block and stained with hematoxylin and eosin (H&E) to confirm the histologic diagnosis and to define the purity of tumor or AH cells. For tissues in which the AH or EC area comprised ≥10% of the slide and the slide accounted for <20% of necrosis, the tissue sample was included in the DNA analysis. DNA was extracted from tissue samples using a commercial DNA extraction kit (QIAamp Tissue Kit; Qiagen, Hilden, Germany). DNA was prepared as described previously [26].

DNA from each tissue block was subjected to bisulfite methylation analysis. The DNA was treated with bisulfite using a CpGenome Universal DNA Modification Kit (Millipore, Bedford, MA) as described previously [26]. TaqMan-based QMSP (MethyLight) was performed after bisulfite treatment of denatured genomic DNA [27]. The methylation status of the candidate genes *AJAP1*, *HS3ST2*, *SOX1*, *PTGDR*, and *LMX1A* was tested. The primer sequences and cover promoter region of *AJAP1*, *HS3ST2*, and *PTGDR* are summarized in Table S1. The master mix and primers for *SOX1* and *LMX1A* were purchased from iStat Biomedical Co. Ltd. The collagen type II α1 gene (*COL2A*) was used as an internal reference gene by amplifying non-CpG sequences. Each sample was analyzed in duplicate. *In vitro* Genome Universal Methylated Genomic DNA (Millipore) was used as a positive control because it is considered to represent 100% methylation of each gene. QMSP was performed in a total volume of 20 μL that contained 2 μL modified template DNA, 1 μL 20× custom TaqMan reagent, and 10 μL LightCycler 480 Probes Master (Roche, Indianapolis, IN). The samples were subjected to an initial incubation at 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s, and annealing and extension for 1 min at the appropriate temperature, and then detected using the LightCycler 480 Real-Time PCR System