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GATA6 expression promoted by an active enhancer may become a molecular marker in endometriosis lesions

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Abstract

PROBLEM: Genome-wide profiling of DNA methylation in endometriotic cells have shown a distinct facet of epigenetic backgrounds, however, specific DNA methylation sites responsible for aberrant gene expression in endometriosis were unknown. Are there specific endometriosis-associated DNA methylations that can be used as molecular markers in endometriosis lesions?

METHOD OF STUDY: This study used endometriotic tissues from the chocolate cyst lining of the ovaries of patients with endometriosis, and endometrial tissues from disease-free patients. For analysis, stromal cells were collected from endometrial and endometriotic tissues. Using endometrial

cells as control, differentially methylated cytosine-phosphate-guanine (CpG) characteristic in endometriotic cells were extracted. Among these CpGs, we focused on a stretch of hypomethylated CpGs within GATA6 gene and examined the potential role as enhancer in endometrotic cells and tissues.

RESULT(S): We identified a stretch of hypomethylated CpGs within the GATA6 gene body in endometriotic cells. Because GATA6 mRNA was highly expressed in endometriotic cells but not in endometrial cells, we then hypothesized that the hypomethylated sequence may function as an enhancer in GATA6 gene expression. Chromatin immunoprecipitation analysis predicted the presence of active enhancer within the gene body sequence in endometriotic cells. Immunohistochemistry showed a positive staining of GATA6 in ovarian chocolate cysts, while in endometrial tissues and in some peritoneal tissues with endometriosis, GATA6 staining was at a marginal level.

CONCLUSION(S): This is the first implication showing a link between an aberrant DNA methylation of *cis* element and gene expression in endometriosis. GATA6 expression may become a molecular marker to diagnose endometriosis lesions.

Key words: endometriosis, endometriosis-associated DNA hypomethylation, active enhancer, GATA6 expression, molecular marker

1. Introduction

Endometriosis is an estrogen-dependent, inflammatory disease characterized by the presence of endometrium-like tissue primarily on the pelvic peritoneum and ovaries. Its prevalence in approximately 10% of women of reproductive age adversely affects their quality of life because of chronic pelvic pain and infertility[1,2]. Approximately 40% of infertile women have endometriosis[3-6], and approximately 1% of endometriotic tissues progresses to malignancy[7,8]. Although many theories, including the retrograde menstruation theory[9] and the coelomic metaplasia theory[10] have been proposed to explain the pathogenesis of endometriosis, they all still remain to be demonstrated.

There is accumulating evidence suggesting epigenetic aberrations play a role in the pathogenesis and/or the pathophysiology in endometriosis[11-13]. Epigenetic regulation, including DNA methylation and histone modifications, are responsible for a number of gene transcriptions that distinguish the various cell types and the states of diseases, including cancer and neurological disease[14-16].

In endometriosis, a number of aberrant gene expressions have been demonstrated[17,18], and these aberrations may be related to aberrant DNA methylations. We hypothesized that genome-wide profiling of DNA methylation could show a distinct profile of epigenetic backgrounds in endometriosis that can be used as molecular markers. To find DNA methylation signatures that can be used as molecular marker in endometriosis, we challenged to extract CpG loci with aberrant methylation associated with gene expression in endometriotic cells. Genome-wide DNA methylation analysis in endometriotic cells reported the genome-wide DNA methylation profile[19,20]. Consistent with their analysis, we observed that the overall methylation profile in endometriotic cells was highly similar to that in endometrial cells. In our genome-wide DNA methylation analysis, we extracted only 1,811 CpGs (0.38%), which showed more than 10-fold difference in the methylation rate, were extracted. The similarity in endometrial and endometriotic cell methylation seemed to support the retrograde menstruation theory for the pathogenesis of endometriosis[9]. Among these CpGs extracted, we found a cluster of hypomethylated CpGs within GATA 6 gene body in endometriotic cells as reported[19,20]. GATA6 is a zinc finger-containing transcription factor that has been shown to play critical roles in cell lineage determination during early embryonic development and organ formation[21]. Loss of GATA6 has been implicated in ovarian cancer development[22]. From our observation that GATA6 was highly expressed in endometriotic cells as reported[19,20], we hypothesized and searched

for the presence of *cis*-acting element within the gene body. Here we show for the first time the presence of active enhancer within the hypopethylated gene body in endometriotic cells. More importantly, we suggest a distinct pattern of GATA6 expression as a molecular marker to diagnose endometriosis lesions.

2. Materials and Methods

Patients

The Institutional Review Boards of the Tottori University Faculty of Medicine approved this project. We obtained informed consent from all patients. Endometrial and endometriotic tissues were obtained from patients as described [23-26]. In brief, endometrial specimens of 12 patients without endometriosis and endometrial cancer were used. None of the patients had received hormonal treatment for at least 2 years prior to the surgery. Eendometrial tissues were obtained from uteri of women with menstrual cycle who underwent hysterectomy for uterine leiomyoma during the proliferative or secretory phase. The chocolate cyst lining of the ovaries of 12 patients with endometriosis was the source of endometriotic tissue collected during the follicular or luteal phase. In a preliminary immunohistochemistry, 3 peritoneal

specimens included the endometriotic lesions were collected from the patients without chocolate cysts in laparoscopic operation.

Preparation of endometrial and endometriotic stromal cells

Stromal cells were collected from endometrial and endometriotic tissues[23-26]. In brief, endometrial and endometriotic tissues were minced and digested with 0.5% collagenase in Dulbecco's modified Eagle medium (DMEM/F12) (Wako, Osaka, Japan). The dispersed cells were filtered through a 70 μm nylon mesh to remove the undigested tissue pieces containing the glandular epithelium. The filtered fraction was separated further from epithelial cell clumps by differential sedimentation at unit gravity. The medium containing stromal cells was filtered through 40μm nylon mesh. Final purification was achieved by allowing stromal cells to adhere to culture dishes within 30 minutes after seeding. To confirm the purity of the isolated stromal cells, immunocytochemical analysis was performed using cytokeratin (DAKO Corp., Kyoto, Japan) as a marker of epithelial cells, vimentin (DAKO Corp.) as a marker of stromal cells, CD14 (Nichirei, Tokyo, Japan) as a marker of activated macrophages, and factor VIII F8/86 (DAKO Corp.) as a marker of endothelial cells. The microscopic examination showed that the purity of the stromal cells was more than 98%. Unless otherwise indicated, cells were cultured in DMEM/F12 (Wako) supplemented with 10% fetal calf serum (FCS) (Thermo Fisher,

Tokyo, Japan) at 37° C in 5% CO_2. At the first passage, endometrial and endometriotic stromal cells were plated at a density of $5x10^5$ cells/60mm dish and incubated to reach a confluence of 80~90% for the preparation of RNA and cell lysate.

Extraction of differentially methylated CpG

Cellular DNAs were prepared from stromal cells from four endometrial (two from proliferative phase and two from secretory phase) and four endometriotic tissues (two from follicular phase and two from luteal phase), using the QIAamp DNA kit (QIAGEN, Tokyo, Japan). Cellular DNAs from endometrial cells were used as control. Following the treatment with bisulfite, DNA was assayed on Infinium Human Methylation450 Beadchip (illumina, Tokyo, Japan)[27]. GeneSpring GX (Agilent, Tokyo, Japan) was used to analyze samples, and CpGs showing the difference in the rate of methylation were extracted.

GATA6 mRNA expression in endometrial and endometrotic cells

Total cellular RNA was prepared from **s**tromal cells collected from endometrium and endometriotic tissues, using the RNeasy Plus kit (QIAGEN, Tokyo, Japan). Single-stranded cDNA was prepared from 2 μg of total cellular RNA in 20 μl of reaction mixture containing 2.5 mM each of dNTP, 20

units of PrimeScript II RTase (Takara, Kyoto, Japan), 20 units of RNase inhibitor (Takara, Kyoto, Japan) and 20 μM of oligo-dT_{16} primer at 42° C for 30 min. Then, 1.5 μl of the cDNA reaction mixture was subjected to PCR amplification in 25 μl Multiplex PCR mixture (Takara, Kyoto, Japan) containing 50 *p*mol each of the respective forward and reverse primers: GATA6-F1 (5'-AGTGCAGACCTGCTGGAGGA-3' in Exon II) and GATA6-R2 (5'-ACTTGAGCTCGCAGTTCTCG-3' in Exon VII). The conditions for PCR were 30 sec at 94° C, 10 sec at 64° C, and 30 sec at 72° C for 35 cycles. At the end of the PCR cycles, 6 μl of the reaction mixture was removed and subjected to electrophoresis on a 1.8% agarose gel in Tris-borate-EDTA buffer at a constant voltage of 100 V. A separated 592 bp sequence (NM_005257) stained with ethidium bromide was visualized under UV light. As internal control, β-tubulin mRNA was assayed in parallel.

Promoter analysis of GATA6 mRNA expression

Using forward primers for the untranslated first exons, 1a (5'-GCTGTTTGTTTAGGGCT-CG-3') or 1b (5'-GCGGTTTCGTTTTCGGGGAC-3') and a reverse primer in Exon II (5'-AAGGGATGCGAAGCGTAGGA-3'), promoter analysis of GATA6 mRNA expression

was assayed[28]. A 511bp sequence from the 1a and a 319 bp sequence from the 1b were amplified. As an internal control, β-tubulin mRNA was assayed in parallel.

Quantitative analysis of GATA6 mRNA expression

Single-stranded cDNAs were prepared from randomly picked up 8 endometrial and 8 endometriotic cells, and were subjected to the SYBR Green real-time PCR in triplicate according to the manufacturer's protocol (Takara, Kyoto, Japan). ABI PRISM 7900HT System was used.

Chromatin immunoprecipitation (ChIP) analysis and non-coding RNA expression ChIP analysis was performed according to the manufacturer's protocol (Simple ChIP, Cell Signaling Tech, Danvers, MA, USA). In brief, endometrial and endometriotic cells were cross-linked in 1% formaldehyde, and the fragmented chromatin was prepared using a digestion with micrococcal nuclease. Following chromatin immunoprecipitation and DNA purification, relative concentrations of GATA6 intron II-upstream, GATA6 intron II-middle, GATA6 intron II-downstream and GATA6 intron III-upstream sequences (see Fig.3A) were assessed by semi-quantitative PCR. Primer sets for GATA6 intron II-upstream 254bp (GATA6-ChIP-F1: 5'-TGGTCCCAGGAAGGATTTGCAGGC-3',

GATA6-ChIP-R1: 5'-ACCCAAAGTAGGGTC-TGCGATGG-3'), GATA6 intron II-middle 321bp (GATA6-ChIP-F3: 5'-TGGTACCACA-CACAGAAAGACAGG-3', GATA6-ChIP-R3: 5'-TACCTCTTTCCCAAACCCACCAGG-3') and GATA6 intron II-downstream 193bp (GATA6-ChIP-F2: 5'-ACGACGTAC-TGTAATGATGATGG-3', GATA6-ChIP-R2: 5'-GACCATTTGTCCAATACTTCTGC -3'). GATA6 intron III-upstream 315bp (GATA6-ChIP-Int-III-F1: 5'-GAAGCCAAGATA-GCCTTTCCC-3', GATA6-ChIP-Int-R1: 5'-GGACGAATGGTATAAGTGAGC-3'). The conditions for PCR were 30 sec at 94° C, 8 sec at 60° C, and 30 sec at 72° C for 36 cycles for GATA6 intron II-upstream and GATA6 intron II-middle. For GATA6 intron II-downstream and GATA6 intron II-PCR, the conditions for PCR were 30 sec at 94° C, 8 sec at 54° C, and 30 sec at 72° C for 36 cycles. Antibodies used were anti-Histone H3K4me1 (#5326, Cell Signaling Tech, Tokyo, Japan) and anti-Histone H3K27ac (#4353, Cell Signaling Tech, Tokyo, Japan). As positive and negative control, anti-Histone H3 (#4620, Cell Signaling Tech, Tokyo, Japan) and normal rabbit IgG (#2729, Cell Signaling Tech, Tokyo, Japan) were used for immunoprecipitation, respectively. Oligo-dT_{16}-primed cDNA preparation mixtures of total cellular RNA, with or without reverse transcriptase, were used for the detection of transcripts from the intron II and the intron III. The primer sets and the PCR conditions employed were the same in the ChIP analysis.

Western blot analysis

Cells were washed with ice-cold phosphate-buffered saline (PBS) and treated with ice-cold 10% trichloroacetic acid for 15 min. Then, the fixed cells were scraped off from dishes and recovered by a centrifugation at 15,000 rpm for 5 min. The resultant pellets were solubilized in 100 μl of sodium dodecylsufate (SDS) sample buffer containing 125mM Tris-HCl (pH6.8), 2.3%(W/V) SDS, 10%(W/V) glycerol, 5%(V/V) 2-mercaptoethanol, and 10 μg/ml bromophenol blue, and then incubated for 5 min in boiling water. Proteins were quantified using BIO-RAD protein assay dye (Bio-Rad Lab, Hercules, CA, USA). The protein samples (20μg per lane) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA), using semidry transfer blot system. To block the non-specific reaction, the PVDF membrane was incubated at room temperature for 1 hr in a solution of PBS with 0.5% Tween 20 (PBS-T) containing 5% non-fat dry milk powder (blocking buffer). The blocked membranes were then incubated overnight at 4°C with anti-GATA6 antibody (H92, Santa Cruz Biotech, CA, USA) in blocking buffer. The blots were washed 4 times with PBS-T, and then incubated with horseradish peroxidase (HRP) -conjugated secondary antibody in blocking buffer at room temperature for 1 hr, followed by the ECL Prime chemiluminescence reaction (GE Healthcare, Little Chalfont, UK). Signals were visualized using image analyzer (LAS-4000, FUJIFILM, Tokyo,

Japan). The membranes were reproved with anti-β-tubulin antibody to confirm the amounts of protein transferred to PVDF membrane.

Immunocyto- and immunohistochemistry

Formalin-fixed and paraffin embedded cells and tissues were cut into a serial tissue sections at a thickness of 4 μm. Following deparaffinization, the sections were immunostained using the streptavidin-biotin alkaline phosphatase complex method according the manufacturers protocol (HISTOFINE SAB-AP (M) Immunohistochemical Staining Kit, Nichirei, Tokyo, Japan). In brief, the section slides were incubated at room temperature for 20 min in blocking serum to eliminate the non-specific binding of primary antibody. The slides were then serially incubated with anti-GATA6 antibody (H92, Santa Cruz Biotech, CA, USA) overnight at 4° C, followed by the secondary antibody. As negative control, the sections were incubated without the primary antibody in parallel. At the end of the incubation with secondary antibody, the sections were washed and subjected to the incubation with streptavidin-alkaline phosphatase reagent. The sections were finally visualized with new fuchsin and counterstained with hematoxylin.

Statistical analysis

Results were analyzed using one-way analysis of variance (one-way ANOVA) followed by Fisher's protected least significant difference test. Values were expressed as means ± standard error of mean (SEM). $p < 0.05$ was considered statistically significant.

3. Results

Extraction of endometriosis-associated CpG methylation

In endometriosis, a number of aberrant gene expressions have been demonstrated [17,18], and these aberrations may be related to aberrant DNA methylations. We then hypothesized that genome-wide profiling of DNA methylation could identify a distinct profile of epigenetic backgrounds that can be used as molecular markers in endometriosis. Using eutopic endometrium-derived stromal cells (endometrial cells) as control, differentially methylated CpGs in chocolate cyst-derived stromal cells (endometriotic cells) were extracted. Human Methylation450 BeadChip analysis identified 1,811 CpGs, which exhibited more than 10-fold difference in the rate of CpG methylation. The 47% (857 CpGs) were hypomethylated, while the remaining 53% (954 CpGs) were hypermethylated.

Extraction of hypomethylated GATA6 gene body

Based on the previous study, we hypothesized the presence of CpG hypomethylation, which is responsible for increased gene expression in endometriotic cells. To address this, we narrowed down the hypomethylated 857 CpGs to 317 using a cut-off value of β 0.25 (Supplemental table1). From the 317 hypomethylated CpGs, we finally extracted a stretch of 14 CpGs within GATA6 gene body, because the difference in the rate of methylation was around 0.9, and this is the largest number of CpGs included in a gene (Supplemental table2). The difference in the β value suggests the presence of two cell population: endometriotic (hypomethylated) and endometrial (hypermethylated) cells. These CpGs are located from the intron II through the upstream of intron III (Fig.1 Top). The rate of methylation in these CpGs was around 0.1 in endometriotic cells, which was in marked contrast to the heavily methylated CpGs in endometrial cells (Fig.1 Bottom).

GATA6 mRNA expression

To further examine GATA6 gene expression, RNA from chocolate cyst-derived endometriotic cells and disease-free eutopic endometrial cells were analyzed by semi-quantitative reverse-transcription PCR. GATA6 mRNA expression was upregulated in the endometriotic cells (Fig.2A). Using

promotor-specific GATA6 primers, GATA6 1a promoter-dependent mRNA expression was demonstrated in endometriotic cells, while the expression was at a marginal level in endometrial cells (Fig.2B). qRT-PCR showed that the expression in endometriotic cells was 50-fold higher than that in endometrial cells (Fig.2C).

ChIP analysis of GATA6 gene body sequence

From the observation above (Fig.2), we hypothesized the presence of *cis*-acting element within the gene body of GATA6 that alters gene expression. ChIP analysis using H3K4me1 and H3K27ac antibodies predicted the presence of active enhancer [29,30] within the hypomethylated sequence in chocolate cyst-derived endometriotic cells (Fig. 3A). Primer sequences were used to analyze four locations in the GATA6 gene: intron II, location 1299-1552 (Fig.3A, a); intron II, location 3869-4099 (Fig.3A, b); intron II, location 5471-5663 (Fig.3A, c); and intron III, location 6240-6554 (Fig.3A, d). The ChIP signal mapped the upstream and the downstream regions of intron II, suggesting the presence of at least two enhancer elements within the gene body sequence (Fig.3A, a and c). Any sign of the ChIP signal was not observed in the intron III sequence (Fig. 3A, d). In endometrium-derived cells, ChIP signal corresponding to H3K4me1 was detectable, but H3K27ac signal was at a marginal level, suggesting the presence of repressed enhancer[29,31] in endometrial cells. Further supporting the

presence of enhancer, non-coding RNA transcribed from the intron II (Fig.3B, a, b and c) was demonstrated both in endometriotic and endometrial cells (Fig.3B), suggesting the expression of enhancer RNA[32].

GATA6 expression in endometrotic cells and tissues

To confirm an increase in GATA6 in endometriotic cells, Western blots were performed on chocolate cyst-derived endometriotic cells and eutopic endometrium-derived cells using an anti-GATA6 and anti-β-tubulin antibodies. Expression of wild type GATA6 was observed in endometriotic cells, but not in endometrial cells (Fig.4A). Immunocytochemistry showed positive staining of GATA6 in endometriotic cell nuclei, while the staining was at a marginal level in endometrial cells (Fig.4B). Immunohistochemistry showed a positive staining of GATA6 in chocolate cysts, and the staining was stronger in the epithelial cells than that in the stromal cells (Fig.4C). In endometrium (Fig.4C) and three peritoneal tissues with endometriosis (Fig.4D), the overall staining was at a marginal level except for some epithelial cell liners in endometrium.

4. Discussion

Aberrant DNA methylation may become a molecular marker to address the pathogenesis and/or pathophysiology in endometriosis[11-13,24,25]. It has been shown that there are differences in methylation profiles in endometriotic cells when compared to eutopic endometrial cells[19,20]. To further assess these differences, we focused on aberrant DNA methylation associated with gene expression in endometriotic cells that have the potential to be used as molecular markers.

To examine the association of the hypomethylated GATA6 gene body (Fig.1) with mRNA expression in endometriotic cells (Fig. 2), we hypothesized the presence of a *cis*-acting element within the gene body sequence. Consistent with our hypothesis, the presence of active enhancer[29,30] within the gene body sequence was predicted in endometriotic cells (Fig.3). In endometrial cells where a marginal expression of GATA6 was observed, the presence of repressed enhancer[31] was predicted (Fig.3). To our knowledge, this is the first observation suggesting a suppressive effect of DNA methylation on enhancer activity.

Enhancers have been accepted as *cis*-regulatory elements that control the timing and location of gene expression. So far, various post-translational modifications of histones have been referred to as enhancer marks. Since Heintzman *et al*. first reported that H3K4me1 and H3K27ac could be enhancer marks to identify the location of putative enhancers[33], there has been accumulating evidence that

H3K27ac combined with H3K4me1 can be used as a tool to identify the location of putative enhancers genome-wide[29,30]. In addition to the active enhancer mark, various combinations of enhancer marks associated with different states of enhancer activity have been reported[29-31]. Our observation predicting the presence of active enhancer within GATA6 intron II in endometriotic cells (Fig.3) is compatible with the accepted criteria for active enhancers. At present, it is not clear whether these predictive enhancer marks are required for enhancer activity or are the consequence of the enhancer's activity[34]. Supporting the presence of enhancers, non-coding RNA known as enhancer RNA (eRNA) has been suggested to play a role in gene regulatory networks[32,35]. In our study, non-coding RNA transcribed from the GATA6 intron II was demonstrated both in endometriotic and endometrial cells (Fig.3B). The observation supports our notion that GATA6 mRNA expression depends on active enhancer within the intron II in endometriotic cells.

GATA6 was highly expressed in endometriotic stromal cell nuclei (Fig.4A) and positive staining of GATA6 was obvious in ovarian chocolate cysts, but not in endometrium (Fig. 4B). Interestingly, GATA6 expression was higher in the epithelial cells than that in the stromal cells. This observation suggests the presence of epithelial cell-specific background of GATA6 expression in chocolate cysts (Fig. 4C). It seems interesting to know the DNA methylation profile in the epithelial cells. Although still a preliminary observation, a marginal expression of GATA6 was observed in three peritoneal

tissues with endometriosis (Fig.4). The observation may imply the presence of heterogeneity in endometriosis lesions. Studies using more peritoneal specimens with endometriosis are underway.

In conclusion, our study suggests that GATA6 gene expression associated with aberrant DNA methylation may be a novel molecular marker for the diagnosis of endometriosis lesions. In addition, the gene body sequence of GATA6 may function as active enhancer under the control of DNA methylation in endometriotic cells (Fig.3). This is the first implication showing a link between an aberrant DNA methylation of *cis* element and gene expression in endometriosis.

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Conflict of interest

The authors disclose no conflicts of interest.

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Figure legends

Figure 1. Hypomethylated CpGs within GATA6 gene body.

(Top) Hypomethylated CpGs within GATA6 gene body. A diagram of the GATA6 gene shows exon II and exon III (white rectangles, distances marked below in bp) and locations of hypomethylated CpGs (vertical lines numbered 1-14). (Bottom) β values and location of 14 CpGs from four endometrial cells and four endometriotic cells; ● : endometrial cells, ○ :endometriotic cells.

Figure 2. GATA6 mRNA expression in endometrial and endometriotic cells.

(A) GATA6 mRNA expression. Single-stranded cDNA was prepared from total cellular RNA and subjected to PCR. A GATA6 cDNA sequence from exon 2 to exon 7 (592 bp in length) was amplified and subjected to 1.8% agarose gel electrophoresis. As internal control, β-tubulin mRNA was assayed in parallel. (B) Promoter analysis of GATA6 mRNA expression: Upper panel schematically shows the 5'-UTR of GATA6 gene. Arrows indicate the location of primers for promoter PCR. Lower panel shows the representative results of RT-PCR using primer sets of exon 1a and 1b.

(C) Quantitative analysis of GATA6 mRNA expression. Single-stranded cDNAs from eight endometrial and eight endometriotic cells were subjected to the SYBR Green real-time PCR in triplicate, using ABI PRISM 7900HT system. Values were expressed as means ± standard error of mean (SEM). $p = 0.00012$ was obtained and considered statistically significant.

Figure 3. Analysis of histone modifications associated with enhancer and non-coding RNA expression.

(A) Analysis of histone modifications associated with enhancer. ChIP analysis using chromatin samples from endometrial and endometriotic cells was performed according to the manufacturer's protocol. Cells were cross-linked in 1% formaldehyde, and fragmented chromatin was prepared using a digestion with micrococcal nuclease. Following ChIP and DNA purification, relative concentration of a (intron II, location 1299-1552), b (intron II, location 3869-4099), c (intron II, location 5471-5663) and d (intron III, location 6240-6554) sequences (Fig. 3A upper panel) was assessed by PCR. Antibodies used are anti-H3K9me1, and anti-H3K27ac. As positive and negative controls, anti-Histone H3 and normal rabbit IgG were used, respectively.

(B) Detection of non-coding RNA. Oligo-dT-primed cDNA reaction mixtures including total cellular RNAs with or without reverse transcriptase, were used for the detection of transcripts from intron II

(a, b, c) and intron III (d). The primer sets and the PCR conditions used were the same in the ChIP analysis.

Figure 4. GATA6 expression in endometriotic cells and tissues.

(A) GATA6 expression. Protein samples from endometrial and endometriotic cells were subjected to western blot analysis. An antibody raised against the C-terminal region of human GATA6 (H92, Santa Cruz Biotech) was used. As control, β-tubulin expression was reproved on the same membrane filter used for GATA6 expression.

(B) Immunocytochemistry of GATA6 expression: endometrial and endometriotic cell sections were stained with anti-GATA6 antibody at a magnification of 4X.

(C) Immunohistochemistry of GATA6 expression. Upper panel shows the staining of endometrium and chocolate cysts. Inserted images are cropped and magnified regions from the images. Lower panel shows the staining of 3 peritoneal tissues with endometriosis at a magnification of 4X.

Figure 1 IZAWA

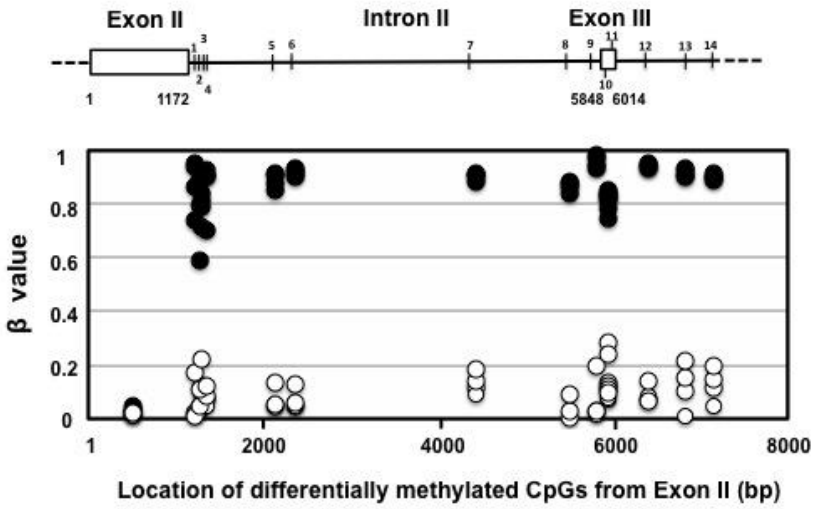

Figure 2 IZAWA

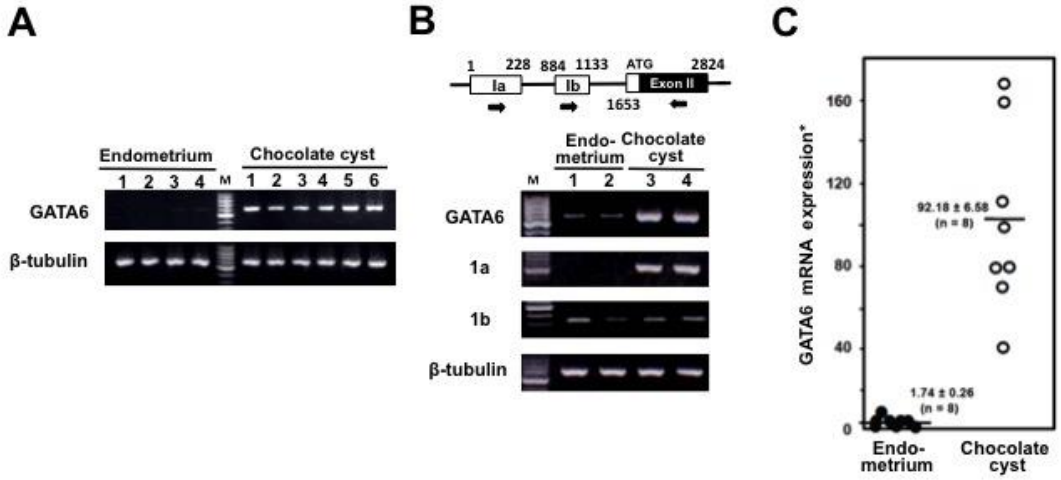

Figure 3 IZAWA

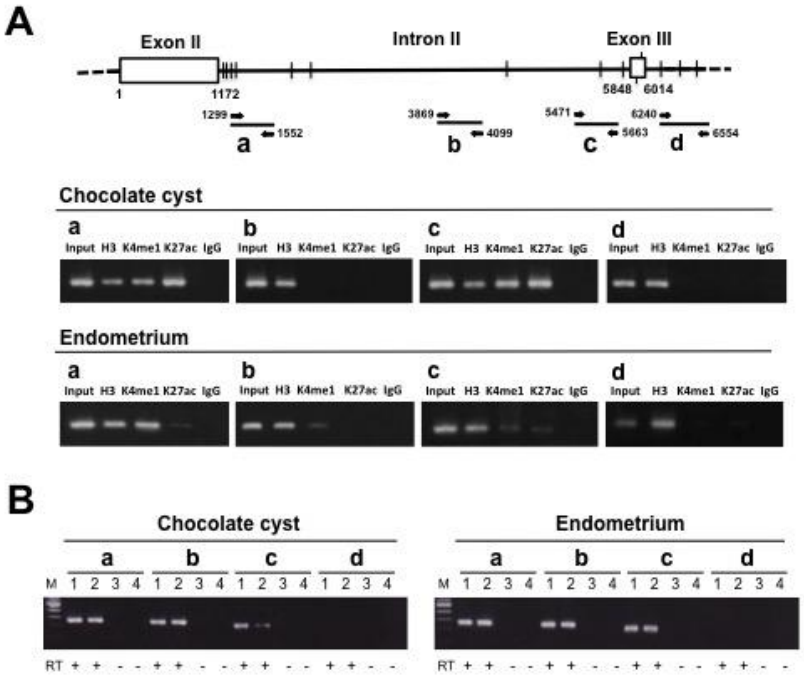

Figure 4 A, B IZAWA

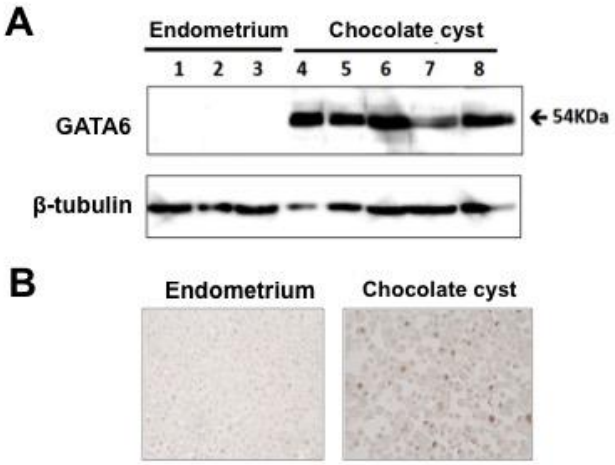

Figure 4 C, D IZAWA