

Fig. 4 Typical chromatograms of spiked urine samples. SIM chromatograms (50 ng ml⁻¹ spiked in urine sample) of BP (A), BP-OH (B), acyl-BP-1 (C), acyl-BP-3 (D), acyl-BP-10 (E), acyl-2OH-BP (F), acyl-3OH-BP (G), and acyl-4OH-BP (H) were monitored at *m/z* 182, 184, 213, 227, 241, 197, 198, and 121, respectively.

Table 1 Analytical figures of merit of HF-LPME with *in situ* derivatization and GC-MS

	LOD/ ng ml ⁻¹	LOQ/ ng ml ⁻¹	Linear range/ ng ml ⁻¹	Correlation coefficient, <i>r</i>
BP	0.02	0.1	0.1 – 100	0.999
BP-OH	0.05	0.2	0.2 – 100	0.999
2OH-BP	0.02	0.1	0.1 – 100	0.995
3OH-BP	0.02	0.1	0.1 – 100	0.998
4OH-BP	0.02	0.1	0.1 – 100	0.997
BP-1	0.02	0.1	0.1 – 100	0.998
BP-3	0.01	0.05	0.05 – 100	0.996
BP-10	0.02	0.1	0.1 – 100	0.996

LOD, Limit of detection (*S/N* = 3); LOQ, limit of quantification (*S/N* > 10)

Determination of BPs in human urine samples

Urine samples from fourteen healthy volunteers (five females and nine males) were analyzed using the present method. BP, 2OH-BP and 3OH-BP were not detected in the human urine samples. In contrast, BP-OH and BP-3 were detected in all urine samples in the range of 0.27 to 10.0 ng ml⁻¹ and 0.36 to 6.91 ng ml⁻¹, respectively (Table 3). Trace amounts of BP-1, BP-10, and 4OH-BP were detected in some samples.

The Ministry of the Environment has published an annual report, "Chemicals in the Environment, FY2005," which contains the results of an environmental survey and monitoring of chemicals. The Ministry of the Environment has also conducted indoor-air monitoring to determine BPs.³³ The report states that BP and BP-3 were determined frequently in the 68 indoor air samples analyzed. Actually, BP and BP-3 were detected in 67 indoor air samples. Taking the frequent detection of BP-3 into consideration, it was reasonable that BP-3 was detected in all human urine samples. Meanwhile, BP, which was also detected in indoor air samples, was metabolized to BP-OH, which was detected in all human urine samples.

The combination of HF-LPME with *in situ* derivatization and GC-MS led to the successful determination of trace amounts of BPs in human urine samples. We have previously reported on

Table 2 Recoveries of BPs in human urine samples

	10 ng ml ⁻¹ spiked		50 ng ml ⁻¹ spiked	
	Recovery, %	RSD, %	Recovery, %	RSD, %
BP	101.5	5.8	99.5	5.7
BP-OH	97.5	6.0	99.7	3.4
2OH-BP	97.9	1.6	100.8	3.0
3OH-BP	97.9	1.5	100.8	3.9
4OH-BP	97.8	2.1	100.2	4.2
BP-1	106.7	7.7	101.5	3.4
BP-3	93.1	8.4	99.5	4.6
BP-10	95.8	7.6	96.3	7.7

The recoveries and RSD were also examined by replicate analysis (*n* = 5) of human urine samples.

Table 3 Concentrations of BPs in human urine samples

Volunteer	Concentration of BPs/ng ml ⁻¹							
	BP	BP-OH	2OH-BP	3OH-BP	4OH-BP	BP-1	BP-3	BP-10
A	N.D.	0.27	N.D.	N.D.	N.D.	0.32	1.23	N.D.
B	N.D.	0.70	N.D.	N.D.	N.D.	N.D.	0.73	N.D.
C	N.D.	2.48	N.D.	N.D.	N.D.	0.54	1.56	N.D.
D	N.D.	5.16	N.D.	N.D.	N.D.	N.D.	2.59	N.D.
E	N.D.	6.36	N.D.	N.D.	N.D.	N.D.	1.02	N.D.
F	N.D.	4.10	N.D.	N.D.	N.D.	N.D.	0.55	0.56
G	N.D.	10.0	N.D.	N.D.	N.D.	N.D.	0.48	0.71
H	N.D.	2.65	N.D.	N.D.	N.D.	N.D.	0.45	N.D.
I	N.D.	1.37	N.D.	N.D.	N.D.	1.15	4.29	0.94
J	N.D.	0.78	N.D.	N.D.	N.D.	0.75	4.91	N.D.
K	N.D.	0.70	N.D.	N.D.	N.D.	N.D.	0.36	N.D.
L	N.D.	0.94	N.D.	N.D.	N.D.	1.91	1.67	1.07
M	N.D.	2.11	N.D.	N.D.	0.15	3.13	6.91	1.31
N	N.D.	4.14	N.D.	N.D.	N.D.	N.D.	2.25	N.D.

N.D. indicates not determined.

the analysis of BPs in a human urine sample by SBSE-TD-GC-MS.¹⁶ In order to improve the sensitivity and cost performance,

we also developed miniaturized HF-LPME for BP analysis.³² In that study,³² five kinds of BPs (BP, BP-OH, 2OH-BP, BP-3, and BP-10) could be analyzed without derivatization. However, an even wider variety of BP-related compounds should be analyzed to reveal the extent of exposure to BPs. Using our proposed improvement method with *in situ* derivatization, BP-1, 3OH-BP, and 4OH-BP were detected, although they could not be detected when *in situ* derivatization was not performed. Moreover, previous GC conditions should be modified to improve the separation of BPs.

This work is the first to determine trace amounts of BPs in human urine samples using HF-LPME with *in situ* derivatization and GC-MS. The proposed method has many practical advantages, including simplicity of the extraction method, the use of a small volume of organic solvent for extraction, and high sensitivity, and is sufficiently applicable to analyses of human urine samples. The proposed method is expected to have potential applications in human urine samples.

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Aberrant DNA methylation status in human uterine leiomyoma

Yoshiaki Yamagata¹, Ryo Maekawa¹, Hiromi Asada¹,
Toshiaki Taketani¹, Isao Tamura¹, Hiroshi Tamura¹, Jun Ogane²,
Naka Hattori³, Kunio Shiota², and Norihiro Sugino^{1,4}

¹Department of Obstetrics and Gynecology, Yamaguchi University Graduate School of Medicine, Minamikagushi 1-1-1, Ube 753-8503, Japan

²Laboratory of Cellular Biochemistry, Animal Resources/Veterinary Medical Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

³Institute of Life Science, Ajinomoto Co., INC., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210-8680, Japan

⁴Correspondence address. Tel: +81 836 22-2286; Fax: +81 836 22-2287, E-mail: sugino@yamaguchi-u.ac.jp

ABSTRACT: Aberrant DNA methylation has been implicated in tumorigenesis. This study was undertaken to establish the genome-wide DNA methylation profile in uterine leiomyomas and to investigate whether DNA methylation status is altered in uterine leiomyomas. For this purpose, restriction landmark genomic scanning (RLGS) was performed on a paired sample of leiomyoma and adjacent normal myometrium. The RLGS profile revealed 29 aberrant methylation spots (10 methylated and 19 demethylated) in leiomyoma in comparison with myometrium. One of the differently methylated genomic loci was newly identified as GS20656 from the human genome sequence database. In 9 of the 10 paired samples, the DNA methylation levels of the first exon of GS20656 were significantly lower in leiomyoma than in myometrium, suggesting the existence of a genomic locus under epigenetic regulation in uterine leiomyomas. Unexpectedly, DNA methyltransferase 1 (DNMT1) and DNMT3a mRNA expression levels were higher in leiomyoma than in myometrium. These facts suggest that other epigenetic factors besides DNMT are involved in local changes of DNA methylation at genome loci. The present study indicates not only aberrant genome-wide DNA methylation status in uterine leiomyomas but also the existence of a genomic locus that is differently methylated between normal myometrium and uterine leiomyoma.

Key words: RLGS / DNA methylation / leiomyoma

Introduction

Uterine leiomyomas are the most common tumors in the female genital tract. Approximately 20–25% of women of reproductive age are afflicted with this disease (Vollenhoven *et al.*, 1990). Uterine leiomyomas cause hypermenorrhea, infertility, miscarriage, etc. and in some cases uterine leiomyomas severely affect a woman's daily life. Despite the high prevalence rate and distressing effect on reproductive women, the pathogenesis of uterine leiomyomas remains unclear. Women with African origin, high body mass index, early menarche, hypertension, history of pelvic inflammatory disease and meat intake, etc. are at greater risk for uterine leiomyoma, whereas women with contraceptive use, smoking, parous and green vegetables intake are at lower risk (Chiapparino *et al.*, 1999; Faerstein *et al.*, 2001a, b; Ryan *et al.*, 2005). These findings suggest that uterine leiomyomas develop not only from inherited genomic abnormalities but also from unfavorable environmental exposure. However, the mechanism of uterine leiomyoma formation and development remains unknown.

Recent microarray analyses have provided much information about mRNA expression in uterine leiomyomas and normal myometrium

(Tsibris *et al.*, 2002; Ahn *et al.*, 2003; Wang *et al.*, 2003; Weston *et al.*, 2003; Catherino *et al.*, 2004). Epigenetic mechanisms including DNA methylation and histone modification are known to play key roles in transcriptional regulation. DNA methylation is involved in various developmental processes through the silencing, switching and stabilizing of genes (Li, 2002; Shiota and Yanagimachi, 2002; Shiota, 2004). Aberrant DNA methylation has also attracted researchers' attention in the study of the mechanism of tumorigenesis. Abnormal DNA methylation of the key gene known as a tumor suppressor is involved in carcinogenesis (Ushijima and Okochi-Tanaka, 2005). Interestingly, global hypomethylation and imbalanced expression of DNA methyltransferases (DNMT), which add a methyl group to the cytosine ring to form methyl cytosine (Smith, 1994), were found in uterine leiomyomas (Li *et al.*, 2003). Furthermore, we recently found aberrant DNA methylation at estrogen receptor- α (ER- α) in uterine leiomyomas (Asada *et al.*, 2008), which suggests that epigenetic alteration of the ER- α gene is involved in their pathogenesis. Therefore, a study of both epigenetics and DNA methylation dependent gene regulation are now essential for investigating the molecular mechanisms involved in the formation and development of uterine leiomyomas.

DNA methylation at the CpG dinucleotides is catalyzed by DNMT. Several DNMTs exist. DNMT1 is responsible for accurately replicating genomic DNA methylation patterns during cell division in mammalian cells (Liu et al., 1998). On the other hand, DNMT3a and DNMT3b are thought to catalyze *de novo* methylation of DNA (Hsieh, 1999). DNMT1, DNMT3a and DNMT3b have also been shown to cooperatively maintain DNA methylation (Ting et al., 2004).

Because mammalian genomes have numerous tissue-dependent differentially methylated regions (T-DMRs) and developmental processes are associated with changes of epigenetics in genome-wide T-DMRs (Cho et al., 2001; Imamura et al., 2001; Shiota, 2004), epigenetic abnormalities may be present in uterine leiomyomas. To date, genome-wide DNA methylation has not been compared between normal myometrium and uterine leiomyoma tissue. The present study was, therefore, undertaken to establish the genome-wide DNA methylation profile of uterine leiomyomas.

Materials and Methods

This study was reviewed and approved by the Institutional Review Board of Yamaguchi University Graduate School of Medicine. Informed consent was obtained from the women before collection of any samples for this study.

Tissue samples

Specimens of uterine leiomyoma and corresponding normal myometrium were obtained from 20 premenopausal women, from 37 to 53 (mean 47.2) years of age, who underwent total hysterectomy. All patients were Japanese. The profile of the samples used in this study is shown in Table I. Women who had other gynecological diseases were excluded. Tissues were taken immediately after removal of the uterus, immersed in liquid nitrogen and stored at -80°C until further processing. Tissue sections of each sample were examined under light microscopy after hematoxylin and eosin staining to confirm the pathologic nature of the sample.

Restriction landmark genomic scanning

Restriction landmark genomic scanning (RLGS) was performed on a paired sample of uterine leiomyoma and adjacent normal myometrium (Case 1 in Table I) for screening genome-wide DNA methylation status. Genomic DNA was extracted from the tissues, and RLGS was performed as described previously using the combination of restriction enzymes, NotI-PvuII-PstI (Shiota et al., 2002; Kremenskoy et al., 2003; Hattori et al., 2004a). To block non-specific labeling, genomic DNA was treated with Klenow fragment (TAKARA, Otsu, Japan) in the presence of dGTP, dCTP, ddATP and ddTTP (TAKARA). DNA was digested with NotI as a landmark enzyme (Nippongene, Toyama, Japan), and the resulting cohesive ends were labeled with Sequenase version 2.0 (USB, NE, USA) in the presence of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ and $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$ (Amersham-Pharmacia, Buckinghamshire, UK), digested with PvuII (Nippongene) and then subjected to the first dimension electrophoresis in a 0.9% agarose disc gel for 23 h at 230 V. After the DNA fragments were treated with PstI (Nippongene) in the disc gel, the resulting DNA fragments were separated in second dimensional 5% polyacrylamide gel for 20 h at 150 V. The gel was dried onto chromatography paper (Whatman, Maidstone, UK) and exposed to X-ray film (Kodak, XARS, Eastman Kodak, NY) for 2–3 weeks at -80°C . The profiles were replicated at least twice.

Spot identification by virtual image RLGS

To identify sequences on the spot that are differently methylated between leiomyoma and normal myometrium, we applied virtual image RLGS

Table I Profile of the samples used in this study

Patients	Age	Location of leiomyoma	Diameter of the leiomyoma (cm)
Case 1	46	Intramural	15
Case 2	50	Intramural	3
Case 3	44	Intramural	8
Case 4	53	Intramural	15
Case 5	49	Intramural	9
Case 6	45	Subserosal	7
Case 7	37	Intramural	8
Case 8	52	Subserosal	9
Case 9	48	Intramural	4
Case 10	48	Subserosal	16
Case 11	50	Subserosal	3
Case 12	49	Intramural	16
Case 13	53	Intramural	7
Case 14	41	Submucosal	5
Case 15	47	Intramural	5
Case 16	48	Submucosal	4
Case 17	48	Intramural	8
Case 18	48	Intramural	6
Case 19	48	Intramural	2
Case 20	46	Submucosal	2

Profiles of the samples used in this study are shown. Cases 1–10 were used for analyses of DNA methylation status of the first exon of GS20656 (Fig. 4A), GS20656 mRNA expression (Fig. 4B) and mRNA expression of DNMT (Fig. 5). Cases 11–20 were used only for analysis of mRNA expression of DNMT.

(vi RLGS). Vi-RLGS software was developed as reported previously (Matsuyama et al., 2003). The human genome sequence was downloaded in masked FASTA format from the GenBank ftp site and processed with the combination of NotI-PvuII-PstI recognition sequences. We selected candidate loci for intensity-changed spots in 'real' RLGS by matching the vi-RLGS and 'real' RLGS profiles. The corresponding sequences were retrieved by clicking the spot on the virtual image and were used as queries for sequence analysis in Ensembl to obtain the surrounding sequence information. By using the sequence information, the primer sets were designed for methylation-sensitive quantitative real-time PCR as described below.

Methylation analysis based on real-time PCR

Methylation status at several intensity-changed spots detected by RLGS was evaluated by using a combination of the methylation-sensitive restriction digestion and quantitative real-time PCR (Heid et al., 1996). Genomic DNA was digested by PstI, and the aliquot was treated subsequently with NotI. The primer sets for PCR were designed to amplify the region that includes the NotI site detected in RLGS analysis. One hundred nanograms of genomic DNA treated with or without NotI was analyzed by real-time PCR with the primers. The amount of undigested DNA both in NotI-treated and -untreated genomic DNA was estimated by real-time PCR with SYBR premix (TAKARA) by using Light Cycler (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. The methylation ratio at GENSCAN00000020656 (GS20656) locus, which was conclusively identified as one of the hypomethylated loci in uterine leiomyoma, was

defined according to the proportion of the amount of undigested DNA in NotI-treated genome to that in the NotI-untreated one. The initial DNA amount in the reaction mixture was normalized with RNaseP control reagent (Applied Biosystems, Foster City, CA, USA). For all samples, at least three independent PCR were repeated. Primer sets of GS20656 locus were shown in Table II.

Sodium bisulfite sequencing

Methylation status in the promoter region of the putative gene of GS20656 was investigated using the sodium bisulfite sequencing method. Sodium bisulfite treatment of genomic DNA and sequencing analysis was carried out as reported previously (Hatton *et al.*, 2004b; Asada *et al.*, 2008). Briefly, 2 µg of genomic DNA was digested with NsiI, denatured by adding 0.3 M of NaOH and incubated for 20 min at 42 °C. After incubation, sodium metabisulfite (pH 5.0) and hydroquinone were added to final concentrations of 2.0 M and 0.5 mM, respectively, and the mixture was further incubated in the dark for 16 h at 55 °C. The modified DNA was purified with a Wizard DNA Clean-Up system (Promega, Madison, WI, USA), and the bisulfite reaction was terminated with NaOH at a final concentration of 0.3 M for 20 min at 42 °C. The solution was then neutralized by adding NH₄OAc (pH 7.0) to a final concentration of 3 M. The ethanol-precipitated DNA was resuspended in water, and the DNA fragment covering the putative promoter region and neighboring NotI site region of GS20656 gene was amplified by PCR using the primers shown in Table II. The PCR products were cloned into pGEM-T Easy Vector (Promega), and 10 or more clones randomly picked from each of the two independent PCRs were sequenced to determine the presence of methylated cytosines.

RT-PCR

Total RNA was extracted from paired samples of uterine leiomyoma and normal myometrium using Isoagen (Wako, Osaka, Japan), and real-time

RT-PCR was performed as reported previously (Asada *et al.*, 2008). RT reactions were performed with ExScript RT reagent kit (TAKARA) according to the manufacturer's protocol. Briefly, 2 µg of total RNA was incubated with 4 µl of 5 × ExScript buffer, 1 µl of dNTP mixture (10 mM each), 1 µl of Random primers (50 µM), 0.5 µl of ExScript RTase (200 U/µl) and 0.5 µl of RNase inhibitor (40 U/µl) in 20 µl of reaction mixture at 42 °C for 15 min, after which the reverse transcriptase was inactivated by heating the samples at 95 °C for 2 min. The complementary DNA (cDNA) was immediately used for PCR. All PCRs were performed using SYBR Premix Ex Taq (TAKARA) and a LightCycler (Roche Applied Science, Basel, Switzerland). Briefly, 2 µl of aliquots containing cDNA were amplified in a total volume of 20 µl containing 4 µl of 5 × SYBR Premix Ex Taq and each of primer sets (0.2 µM) described below.

Primer set I was designed in the putative first exon of GS20656 (Fig. 1) for real-time RT-PCR as shown in Table II, corresponding to a 133 bp fragment. To cover a longer region of the first exon of GS20656 than primer set I covers, primer set II (Fig. 1) was designed as shown in Table II and used for RT-PCR, corresponding to a 250 bp fragment. In line with a previous report (Girault *et al.*, 2003), the primers for DNMT1, DNMT3a and DNMT3b were used for real-time RT-PCR as shown in Table II. For internal controls, TATA box-binding protein (TBP) cDNA or GAPDH cDNA was amplified with each set of primers as shown in Table II. All samples were run in duplicate. Melting curves of the products were obtained after cycling by a stepwise increase of temperature from 55 to 95 °C. At the end of 40 cycles, reaction products were separated electrophoretically on an agarose gel and stained with ethidium bromide for visual confirmation of the PCR products.

Statistical analysis

Differences were examined with Student's *t*-test and χ^2 test using the computer program SPSS version 13.0 for Windows. A value of $P < 0.05$ was considered to be significant.

Table II Primers used in the present study

Gene	Primer	Amplification size (bp)	Method
GS20656	F: 5'-CCTCTCCGCCCTCCCGATGG-3' R: 5'-TGATGTGTGTTTGGTGAGCAAGG-3'		Methylation-sensitive quantitative real-time PCR
GS20656	F: 5'-GGGGTGGTTAATTTAGAGATGATT-3' R: 5'-TTACTCTCCAAAACCAAATACCAAA-3'		Sodium bisulfite sequencing
GS20656	F: 5'-CAGCTGGTCACGTCCTCAC-3' R: 5'-CCAAAGGAGAGGCACAAAAG-3'	133	Real-time RT-PCR (primer set I)
GS20656	F: 5'-CAGCTGGTCACGTCCTCAC-3' R: 5'-CAACAATTGCCTCACAATCG-3'	250	RT-PCR (primer set II)
DNMT1	F: 5'-TACCTGGACGACCCTGACCTC-3' R: 5'-CGTTGGCATCAAAGATGGACA-3'	103	Real-time RT-PCR
DNMT3a	F: 5'-TATTGATGAGCGCACAAAGAGAGC-3' R: 5'-GGGTGTTCCAGGGTAACATTGAG-3'	111	Real-time RT-PCR
DNMT3b	F: 5'-GGCAAGTTCTCCGAGGTCTCTG-3' R: 5'-TGGTACATGGCTTTTCGATAGGA-3'	113	Real-time RT-PCR
TBP	F: 5'-TCCACAGGAGCCAAGAGTGAA-3' R: 5'-CACATCACAGCTCCCCACCA-3'	132	Real-time RT-PCR
GAPDH	F: 5'-AGGTGAAGGTCGGAGTCA-3' R: 5'-GGTCATTGATGGCAACAA-3'	99	Real-time RT-PCR

F, forward primer; R, reverse primer

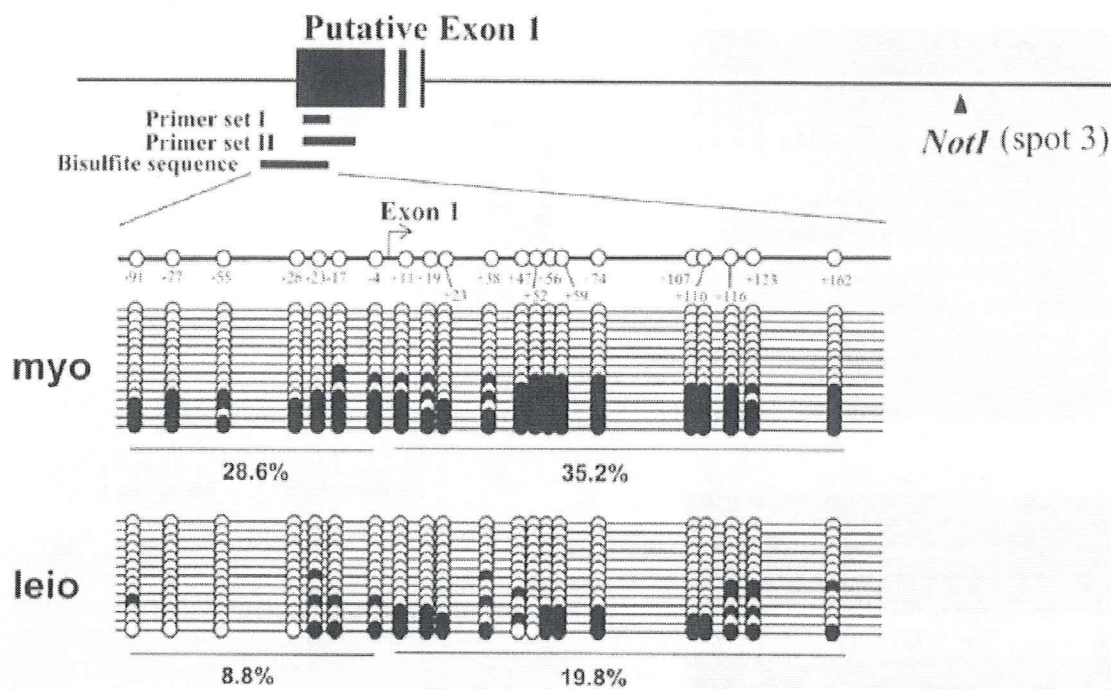


Figure 1 DNA methylation status of the 5'-flanking region of GS20656 in normal myometrium and uterine leiomyoma. The region of the first exon is shown as a thick black bar. The boundary between the first exon and the first intron was blurred. The arrowhead indicates NotI site of the spot #3 in the RLGS profile shown in Fig. 2. The region including the promoter and the first exon (thin black bar) was analyzed by sodium bisulfite sequencing. There are 21 CpG sites between -91 and $+162$ in the promoter and the first exon regions. Methylation status of the individual CpG site was analyzed in the normal myometrium (myo) and the uterine leiomyoma (leio). Open and closed circles indicate unmethylated and methylated CpG status, respectively.

Results

RLGS analysis

A representative RLGS profile of normal myometrium, consisting of ~ 1800 spots, is shown in Fig. 2A. In RLGS profiles, the spots are visible if the corresponding NotI site in the genome is unmethylated or hypomethylated, whereas they are invisible if the NotI site is hypermethylated. Numbered circles indicate the 29 spots in which the intensity of spots was either higher or lower than the corresponding spots in the uterine leiomyoma, indicating that there were 29 altered spots in the uterine leiomyoma compared with the normal myometrium. Figure 2B shows the magnified views of the 29 spots. Nineteen spots indicated by the open arrow heads in the leiomyoma were increased in intensities (# 1–3, 8–12, 15–17, 20, 21, 23 and 25) or newly appeared (# 6, 18, 22 and 27) in comparison with the myometrium, indicating that these spots are demethylated or unmethylated in the uterine leiomyoma compared with the normal myometrium. On the other hand, 10 spots indicated by closed arrow heads in the leiomyoma were decreased in intensities (# 4, 7, 26 and 29) or disappeared (# 5, 13, 14, 19, 24 and 28) in comparison with myometrium, suggesting that these spots are hypermethylated or methylated in the uterine leiomyoma compared with the normal myometrium.

Identification of the gene with altered DNA methylation status

The genomic locus of the spot #3 shown in Fig. 2, which is demethylated in the uterine leiomyoma compared with the normal myometrium, was identified using vi-RLGS. Methylation levels at the spot #3 locus in the uterine leiomyoma and normal myometrium were determined by methylation-sensitive quantitative real-time PCR. The methylation level at the spot #3 locus in the uterine leiomyoma ($13.4 \pm 3.0\%$) was significantly lower than that in the normal myometrium ($52.2 \pm 4.2\%$) (Fig. 3), confirming that this locus is actually hypomethylated in the uterine leiomyoma compared with the normal myometrium. Because the spot #3 locus was not recorded in a known gene coding region in the human genome sequence database, it was considered to be a new putative gene and was named as GS20656 in this study. GS20656 is located in chromosome 15, q23 (AC009434.19.1.177207). Future studies are needed to identify the sequence of other spots that are differently methylated between leiomyoma and normal myometrium.

DNA methylation status of 5'-flanking region of GS20656

The methylation status in the promoter region and the first exon of GS20656 was investigated by sodium bisulfite sequencing (Fig. 1).

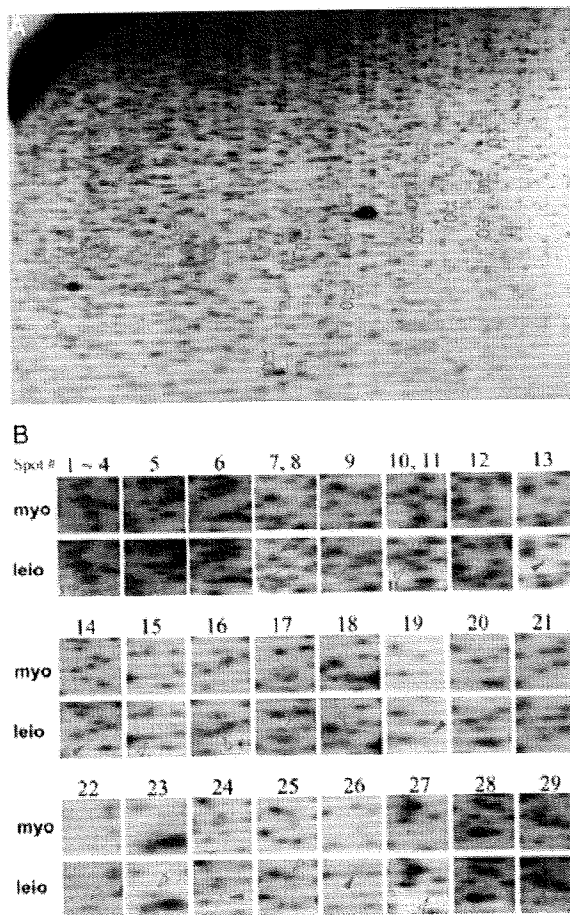


Figure 2 Genome-wide DNA methylation analysis of uterine leiomyoma and the corresponding normal myometrium. (A) Representative RLGS profile of a normal myometrium. Numbered circles indicate the 29 spots in which the intensity of spots was either higher or lower than the corresponding spots in the uterine leiomyoma. (B) Magnified views of the 29 spots in the uterine leiomyoma (leio) and their adjacent normal myometrium (myo). The spots are visible if the corresponding NotI site in the genome is unmethylated or hypomethylated, whereas they are invisible if the NotI site is hypermethylated. Nineteen spots indicated by open arrow heads in the leiomyoma were increased in intensities (# 1–3, 8–12, 15–17, 20, 21, 23 and 25) or newly appeared (# 6, 18, 22 and 27) in comparison with the myometrium. On the other hand, 10 spots indicated by closed arrow heads in the leiomyoma were decreased in intensities (# 4, 7, 26 and 29) or disappeared (# 5, 13, 14, 19, 24 and 28) in comparison with myometrium.

In the promoter region examined, only 8 CpGs (8.8%) in a total of 91 examined CpGs were methylated in the uterine leiomyoma, whereas 28 CpGs (28.6%) in a total 98 examined CpGs were methylated in the normal myometrium. In the first exon examined, 39 CpGs (19.8%) in a total of 196 examined CpGs were methylated in the uterine leiomyoma, whereas 64 CpGs (35.2%) in a total 182 examined CpG sites were methylated in the normal myometrium. In both regions,

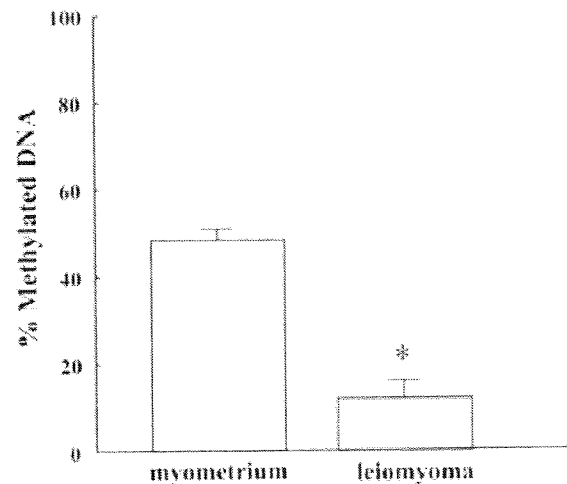


Figure 3 Percentage methylated DNA at the spot #3 locus in the normal myometrium and uterine leiomyoma. DNA methylation levels were determined by methylation-sensitive quantitative real-time RT-PCR as described in the Materials and Methods section. Values are mean \pm SEM of three different experiments. * $P < 0.05$ versus myometrium (Student's *t*-test).

CpGs were significantly hypomethylated in the uterine leiomyoma compared with the normal myometrium ($P < 0.01$, χ^2 test).

Relationship between methylation status and mRNA expression of GS20656

Ten cases (Cases 1–10 in Table I) were analyzed for DNA methylation status and mRNA expression of GS20656. In 9 of the 10 cases examined (all except Case 8), the DNA methylation levels in the first exon of GS20656 were significantly lower in the leiomyoma than in the myometrium (Fig. 4A) ($P < 0.05$, Student's *t* test). In five of these cases, GS20656 mRNA expression was higher in the leiomyoma than in the myometrium (Fig. 4B).

DNMT mRNA expression

DNMT mRNA expression was examined in the same 10 paired samples as shown in Fig. 4. In 9 of the 10 cases examined (all except Case 1), DNMT1 mRNA expression was higher in the leiomyoma than in the myometrium (Fig. 5A). DNMT3a mRNA expression was higher in the leiomyoma than in the myometrium in all 10 cases (Fig. 5B), whereas DNMT3b mRNA expression was higher in the leiomyoma in only two cases (Fig. 5C). Furthermore, DNMT mRNA expression levels were examined in additional 10 paired samples (Cases 11–20 in Table I), and statistical analysis was performed in 20 cases altogether. DNMT1 and DNMT3a mRNA levels were significantly higher in the leiomyoma than in the myometrium (Table III). In 15 of the 20 cases (75%), DNMT1 mRNA expression was higher in the leiomyoma than in the myometrium, and DNMT3a mRNA expression was higher in the leiomyoma in 17 cases (85%) (Table III). Although DNMT3b mRNA levels tended to be lower in the leiomyoma, the difference was not statistically

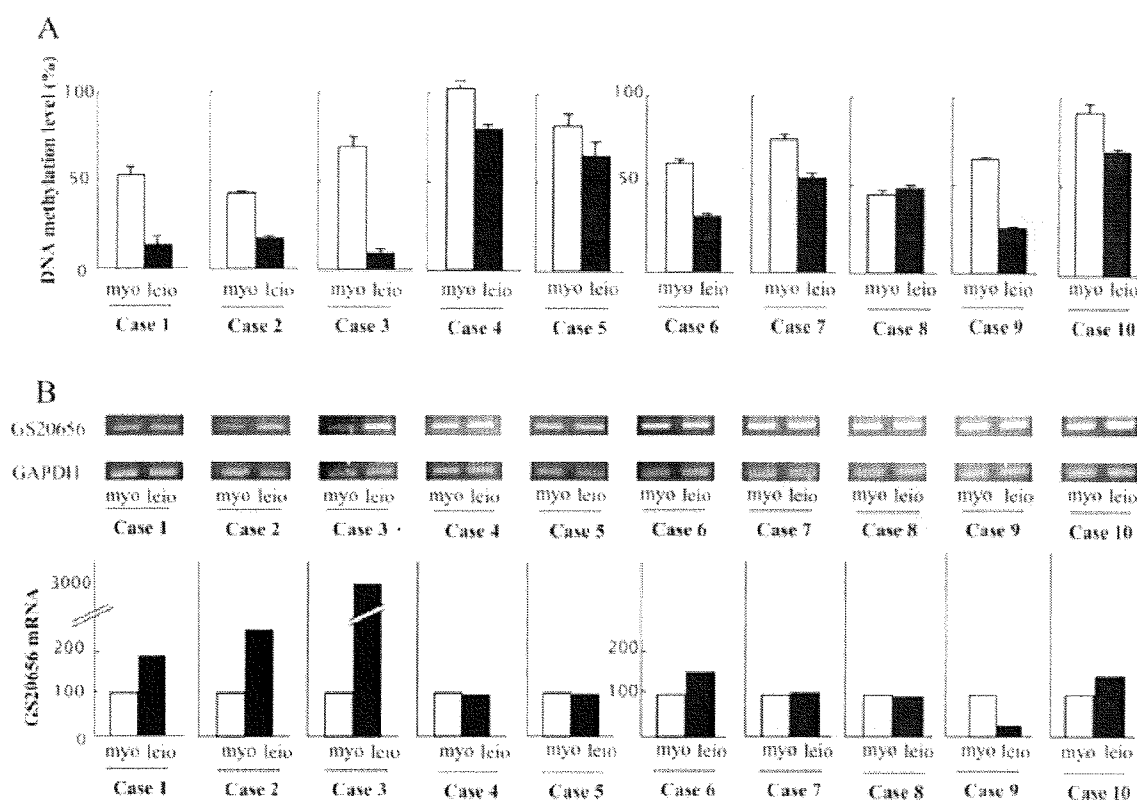


Figure 4 DNA methylation levels of the first exon of GS20656 and mRNA levels of GS20656. Methylation status of the first exon of GS20656 and mRNA expression of GS20656 were examined for 10 paired samples with leiomyoma (leio) and myometrium (myo). **(A)** DNA methylation levels were evaluated by methylation-sensitive quantitative real-time PCR, and expressed as %methylated DNA. Values are mean \pm SD from three different experiments. Methylation levels were significantly different ($P < 0.05$, Student's *t*-test) between myometrium and leiomyoma in all cases except Case 8. **(B)** Representative blotting band of GS20656 and GAPDH by RT-PCR using primer set II, and mRNA expression of GS20656 by real-time RT-PCR using primer set I. Locations of primer sets I and II are shown in Fig. 1. Expression levels in the myometrium were normalized to 100% in each sample.

significant (Table III). There was no correlation between GS20656 mRNA expression and DNMT3b mRNA expression.

Discussion

The present study showed that uterine leiomyoma is associated with alterations of DNA methylation at multiple genomic loci as indicated by a genome-wide DNA methylation analysis using RLGS. The methylation status of 29 NotI sites was altered in uterine leiomyomas in comparison with normal myometrium. Intriguingly, both methylated and demethylated changes occurred at multiple gene loci in uterine leiomyomas. However, since there is a possibility that DNA methylation patterns differ among individuals, further RLGS analyses with larger samples are needed to clarify the detailed difference in genome-wide DNA methylation status between uterine leiomyomas and normal myometrium.

In this study, we identified a new putative gene, GS20656, which showed an aberrant methylation status in uterine leiomyomas compared with myometrium. The fact that the DNA methylation levels of the promoter region and the first exon of GS20656 were

significantly lower in leiomyoma than in the myometrium indicates that GS20656 is regulated by DNA methylation in uterine leiomyomas. These results suggest that there is a gene under epigenetic regulation in uterine leiomyomas.

In the nine cases in which DNA methylation levels of GS20656 were lower in leiomyoma than in myometrium, five cases showed that GS20656 mRNA expression tended to be higher in leiomyoma than in myometrium. In particular, GS20656 mRNA expression is much higher in the cases that have considerable hypomethylation of GS20656 (Cases 1, 2 and 3 in Fig. 4). These results suggest that CpG sites of GS20656 may be associated with mRNA expression level of GS20656. However, it is not surprising that there are cases in which DNA methylation status of GS20656 is not consistent with GS20656 mRNA expression, because DNA methylation may occur heterogeneously and/or gradually and the change in DNA methylation varies among individuals. Our recent report also shows that there are cases in which DNA methylation status of ER- α promoter region is not consistent with ER- α mRNA expression (Asada et al., 2008).

It would be interesting to know the biological role of GS20656. Although the participation of GS20656 in uterine leiomyomas may

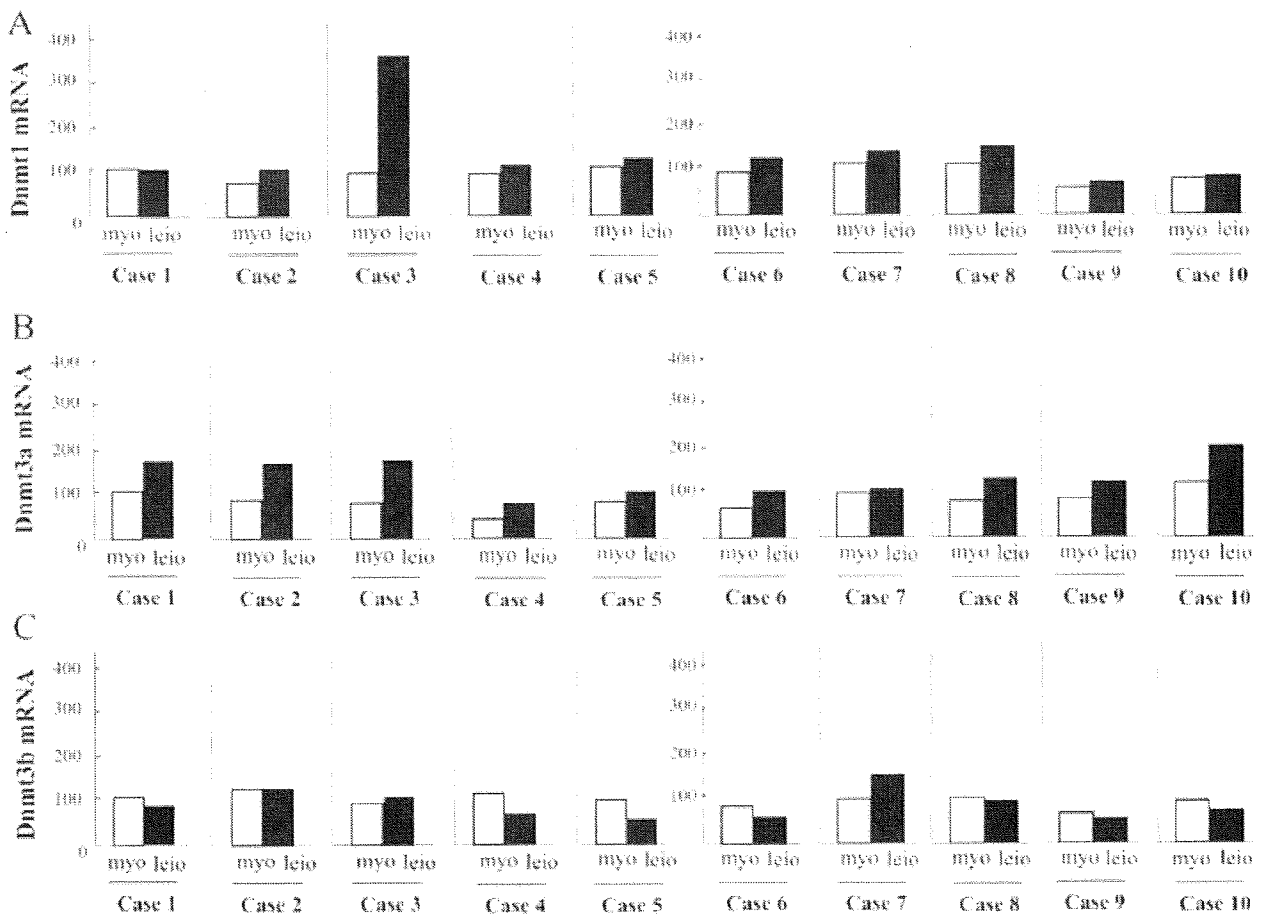


Figure 5 mRNA expression of DNMT in uterine leiomyoma (leio) and myometrium (myo). mRNA levels of DNMT1 (A), DNMT3a (B) and DNMT3b (C) were analyzed by real-time RT-PCR on the same 10 paired samples as shown in Fig. 4.

Table III DNMT mRNA levels in myometrium and leiomyoma

	n	Myometrium	Leiomyoma	Number of myo < leio
DNMT1	20	92.6 ± 5.0	118.6 ± 14.2 ^b	15 (75%)
DNMT3a	20	110.5 ± 12.5	161.9 ± 19.8 ^a	17 (85%)
DNMT3b	20	115.2 ± 8.6	97.2 ± 6.9	6 (30%)

mRNA levels of DNMT1, DNMT3a and DNMT3b were examined for 20 paired samples with leiomyoma (leio) and myometrium (myo) (Cases 1 - 20 in Table I). Values are mean ± SEM.

^aP < 0.01 versus myometrium.

^bP < 0.05 versus myometrium.

be different among the patients, further studies on the biological role of GS20656, including the relationship between GS20656 and clinical features such as tumor growth, phenotypes, hormone-responsiveness, etc. are needed.

Alterations in regional methylation patterns have been associated with silencing of tumor suppressor/DNA repair genes (Ballestar and

Esteller, 2002), transcriptional activation of oncogenes (Feinberg et al., 2002) and loss of imprinting in malignant cells (El-Osta, 2004), suggesting that hypermethylation is involved in the pathogenesis of malignant tumors. It is unclear that hypermethylation or hypomethylation is more important in the pathogenesis of uterine leiomyoma, which is a benign tumor. There are many differences in cell characteristics between malignant tumors and benign ones. Our RLGS analysis showed both methylated and demethylated changes in benign leiomyoma. Cell characteristics of uterine leiomyomas include the potential activities of both methylation and demethylation. This may be supported by the report of Li et al. (2003) that imbalanced methylation status, e.g. coexistence of global hypomethylation and local gene-specific hypermethylation, may exist in leiomyoma. The present result raises the possibility that epigenetic modification of DNA may be involved in the pathogenesis of uterine leiomyomas.

It is of interest that DNA methylation levels of normal myometrium vary among individuals. We previously reported that the DNA methylation status of the ER-α promoter region in normal myometrium varies among individuals, which may represent a physiological change in a certain cell type such as smooth muscle cells in myometrium (Asada et al., 2008). Those results suggest that DNA

methylation seen in the myometrium may be caused by some factors that induce aberrant DNA methylation such as aging, chronic inflammation and possible viral infection (Ushijima and Okochi-Tanaka, 2005).

Li et al. (2003) showed that mRNA expression of DNMT3a and DNMT3b was lower in leiomyomas than in myometrium while most of the leiomyomas had equal or increased expression of DNMT1 compared with the myometrium. The present study showed mRNA levels of DNMT1 and DNMT3a were higher in leiomyomas than in myometrium, whereas there was no significant difference in DNMT3b mRNA expression between leiomyomas and myometrium. An increased DNMT1 expression was consistent in both studies, which may reflect an elevated proliferative activity of leiomyoma cells because DNMT1 is responsible for copying methylation patterns following DNA synthesis (Liu et al., 1998). However, there was a discrepancy in DNMT3a and DNMT3b expression between the report by Li et al. (2003) and the present result. It is hard to clearly explain the inconsistency. This may be attributed to the race-dependent difference. The samples used in the report by Li et al. (2003) were obtained from 16 African-American, 5 Caucasian and 2 Hispanic women, whereas all samples in this study were from Japanese (Asian origin). In fact, it has recently been reported that the promoter which regulates aromatase expression in uterine leiomyomas is different between Japanese women and other race including African-American, Caucasian and Hispanic women (Shozu et al., 2002; Imir et al., 2007). In addition, it is well known that uterine fibroids are more common in African-American women than in white women (Faerstein et al., 2001a, b). However, the findings from Li et al. (2003) and the present study both suggest the possibility that unusual expression of epigenetic factors causes aberrant DNA methylation in uterine leiomyomas.

Hattori et al. (2004a) showed DNA hypomethylation at genomic loci in *Dnmt1*^{-/-} and *Dnmt3a*^{-/-}*Dnmt3b*^{-/-} mouse ES cells compared with wild-type ES cells using an RLGS analysis. Furthermore, Ikegami et al. (2007) showed that G9a deficiency, which impairs histone H3-k9 methylation, caused DNA hypomethylation at the G9a target loci in *G9a*^{-/-} mouse ES cells using RLGS. This indicates that global regulators could contribute to local changes in DNA methylation at genomic loci. It also implies that a combination of epigenetic factors is involved in the maintenance of DNA methylation in cells and tissues. It is apparent from our findings and other reports, further studies are needed regarding the relevance of epigenetic factors to the DNA methylation pattern in uterine leiomyomas.

In conclusion, the present study showed not only aberrant genome-wide DNA methylation status in uterine leiomyomas using RLGS but also the existence of a genomic locus that is differently methylated in normal myometrium and uterine leiomyoma. Epigenetics and DNA methylation-dependent gene regulation may play a role in formation and development of uterine leiomyomas.

Funding

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DNA methyltransferase expression in the human endometrium: down-regulation by progesterone and estrogen

Yoshiaki Yamagata, Hiromi Asada, Isao Tamura, Lifa Lee, Ryo Maekawa, Ken Taniguchi, Toshiaki Taketani, Aki Matsuoka, Hiroshi Tamura, and Norihiro Sugino¹

Department of Obstetrics and Gynecology, Yamaguchi University Graduate School of Medicine, Minamikogushi 1-1-1, Ube 755 8505, Japan

¹Correspondence address. Tel: +81 836 22 2286; Fax: +81 836 22 2287; E-mail: sugino@yamaguchi-u.ac.jp

BACKGROUND: Epigenetic regulation may be involved in modulation of gene expression during the normal cyclic changes of the human endometrium. We investigated expression of DNA methyltransferases (DNMTs) in endometrium during the menstrual cycle and the influence of sex steroid hormones on DNMT in endometrial stromal cells (ESC) in culture.

METHODS: Expression of DNMT1, DNMT3a and DNMT3b was assessed by immunohistochemistry and real-time RT-PCR in endometrial tissue ($n = 42$ women). ESC ($n = 3$ women) were cultured with estradiol and medroxyprogesterone acetate (E + MPA) for 17 days, and DNMT mRNA levels were measured by real-time RT-PCR.

RESULTS: Nuclei of both epithelial and stromal cells immunostained for DNMT1, DNMT3a and DNMT3b during each phase of the menstrual cycle. Tissue levels of DNMT1 and DNMT3a mRNA were significantly lower in the mid-secretory phase than in the proliferative phase ($P < 0.01$). For DNMT3b, the change in mRNA levels showed a similar trend to that for DNMT3a. In ESC culture, DNMT3a and DNMT3b mRNA levels were significantly decreased by E + MPA treatment ($P < 0.01$ and $P < 0.05$, respectively) at Day 8 and Day 17.

CONCLUSIONS: DNMT mRNAs declined in the human endometrium during the secretory phase, and E + MPA down-regulated DNMT3a and DNMT3b mRNAs in ESC in culture. These results suggest that DNMTs have regulatory functions in gene expression that is associated with decidualization.

Key words: DNA methyltransferase / endometrium / endometrial stromal cell / decidualization

Introduction

The human endometrium, which mainly consists of endometrial epithelial cells and endometrial stromal cells (ESC), has cyclic changes in morphology and in function depending on female sex steroid hormone exposure. These cells actively proliferate under estrogen exposure during the proliferative phase and thereafter differentiate under progesterone exposure during the secretory phase. A number of genes are involved in proliferation, differentiation and tissue breakdown in the endometrium under the influence of female sex steroid hormones (Sugino *et al.*, 2002a, 2004; Ace and Okulicz, 2004; Ponnampalam *et al.*, 2004). A great number of genes are up-regulated or down-regulated in the human endometrium during decidualization, which occurs around the time of embryo implantation

(Popovici *et al.*, 2000; Kao *et al.*, 2002; Okada *et al.*, 2003; Riesewijk *et al.*, 2003; Ace and Okulicz, 2004; Mirkin *et al.*, 2005), suggesting the presence of complex mechanisms of gene expression. However, little is known about the molecular mechanisms involved in the regulation of gene expression in the human endometrium.

In the last decades, it has become clear that epigenetic regulation, including DNA methylation and histone modification, plays a key role in transcriptional regulation. DNA methylation occurs at cytosines within CpG dinucleotides that are clustered frequently in regions of ~1–2 kb in length, called CpG islands, in or near the promoter and first exon regions of genes (Esteller *et al.*, 2002; Jones and Baylin, 2002). DNA methylation at the CpG dinucleotides is a post-replication event catalyzed by DNA methyltransferase (DNMT) (Smith, 1994) that adds a methyl group to the cytosine ring to form

methyl cytosine, which establishes normal methylation patterns during embryogenesis and reproduces these patterns during replication of adult cells (Li *et al.*, 1993; Razin and Kafri, 1994). DNA methylation is an important mechanism of epigenetic gene regulation, and is involved in genomic imprinting, X chromosomal inactivation, aging, mutagenesis and regulation of tissue-specific gene expression during development and adult life (Li *et al.*, 1993; Razin and Kafri, 1994; Ohgane *et al.*, 1998; Imamura *et al.*, 2001; Li, 2002; Shiota and Yanagimachi, 2002). Aberrant methylation of CpG islands, located in the 5'-promoter region of genes, is commonly associated with transcriptional inactivation (Nan *et al.*, 1998). Such inactivation is well known in various human cancers, especially in tumor suppressor genes (Ushijima and Okochi-Takada, 2005).

Several DNMTs exist. DNMT1 functions as a 'maintenance' DNMT in mammalian cells and is therefore responsible for accurately replicating genomic DNA methylation patterns during cell division (Liu *et al.*, 1998). On the other hand, DNMT3a and DNMT3b are thought to catalyze *de novo* methylation of DNA (Hsieh, 1999). Recent research also shows that DNMT1, DNMT3a and DNMT3b co-operatively maintain DNA methylation (Ting *et al.*, 2004).

Recently, aberrant expression of DNMTs was observed in endometriosis, which is a non-cancerous ectopic growth of the human endometrium (Wu *et al.*, 2007). Aberrant DNA methylation of the promoter region is involved in aberrant gene expression of steroidogenic factor 1 and estrogen receptor in endometriosis (Xue *et al.*, 2007a, b; Utsunomiya *et al.*, 2008). Furthermore, in the eutopic endometrium of women with endometriosis, reduced expression of HOXA10, which is a transcription factor and plays an important role in uterine receptivity, was found to be due to DNA methylation of the promoter region (Wu *et al.*, 2005). In addition, aromatase expression in ESC is under epigenetic regulation (Izawa *et al.*, 2008). Histone acetylation is involved in differentiation of ESC and endometrial epithelial cells (Sakai *et al.*, 2003; Uchida *et al.*, 2005). These reports led us to hypothesize that epigenetic regulation is involved in the normal cyclic changes of the human endometrium. To test this hypothesis, we investigated changes in the expression of DNMTs in the normal endometrium during the menstrual cycle and the influence of female sex steroid hormones on DNMT expression in ESC.

Materials and Methods

This study was reviewed and approved by the Institutional Review Board of Yamaguchi University Graduate School of Medicine. Informed consent was obtained from the women before collection of any samples for this study.

Tissue samples

Endometrial tissues were collected from hysterectomy specimens or biopsies for histological dating of the endometrium in 42 women with regular menstrual cycles (aged 22–50 years, median 36.9 years). All of the women received no steroid medications. Endometria were dated according to the histological criteria by Noyes *et al.* (1950) and were classified into four different groups: proliferative phase (days 6–14, $n = 14$), early secretory phase (days 15–18, $n = 10$), mid-secretory phase (days 19–23, $n = 11$) and late secretory phase (days 24–28, $n = 7$). Endometrial samples were snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation. For immunohistochemistry, the tissue specimens were fixed in 10% buffered formalin and embedded in paraffin.

Immunohistochemistry

Immunohistochemistry for DNMTs in the endometrium was performed on 4 μm thick paraffin sections mounted on silane-coated glass slides (Dako, Glostrup, Denmark) using anti-DNMT1 monoclonal antibody (IMG-261 mouse; Imgenex, San Diego, CA, USA), anti-DNMT3a polyclonal antibody (RB1852 rabbit, Abgent, San Diego, CA, USA) or anti-DNMT3b polyclonal antibody (RB1906 rabbit; Imgenex), as reported previously (Sugino *et al.*, 1996, 2002b). Briefly, the sections were deparaffinized in xylene and dehydrated through a graded series of ethanol. For antigen retrieval, the sections were autoclaved at 121°C for 15 min. Endogenous peroxidase activities and non-specific binding were then blocked with 1% H_2O_2 and 10% normal rabbit serum (Nichirei, Tokyo, Japan) for DNMT1 or 10% normal goat serum (Nichirei) for DNMT3a and DNMT3b, respectively. The sections were then incubated with the primary antibody diluted 1:50 overnight at 4 $^{\circ}\text{C}$. Parallel control sections were incubated with normal mouse serum or normal rabbit serum (Dako) instead of specific primary antibodies. Biotinylated antimouse antibody (Nichirei) for DNMT1 or biotinylated antirabbit antibody (Nichirei) for DNMT3a and DNMT3b was used as the secondary antibody. After the sections were rinsed in phosphate-buffered saline (PBS), they were incubated in streptavidin-peroxidase complex (Nichirei) for 5 min, rinsed in PBS and then visualized with diaminobenzidine and counterstained with hematoxylin. Dark brown nuclear staining indicated a positive reaction. The histological sections were independently evaluated by three observers, and relative intensities of the signals were estimated at + (weakly positive) to +++ (strongly positive).

ESC culture

For ESC culture, endometrial tissues that were histologically diagnosed as being in the late proliferative phase were used. Tissue samples were obtained from three individuals, and cells from an individual were cultured in triplicate. ESC were isolated as reported previously (Sugino *et al.*, 2000, 2002c). Endometrial tissues were washed with phenol red-free Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Paisley, UK) containing 4 mmol/l glutamine (Invitrogen), 50 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen) and 50 IU/ml penicillin (Invitrogen), and minced into small pieces of $<1\text{ mm}^2$. After the enzymatic digestion of minced tissues with 0.2% collagenase (Sigma, St Louis, MO, USA) in a shaking water bath for 2 h at 37°C , ESC were separated by filtration through a 70 μm nylon mesh. The filtrates were washed three times, and the number of viable cells was counted by trypan blue dye exclusion. The homogeneity of the stromal cell preparation (98%) was verified by immunocytochemistry using an antibody against vimentin, a specific marker of stromal cells. ESC were seeded at 10^5 cells/ cm^2 in 75 cm^2 tissue culture flasks and grown until confluence in phenol red-free DMEM containing glutamine, antibiotics and 10% dextran-coated charcoal-stripped fetal calf serum (FCS) (Biological Industries, Kibbutz Beit Haemek, Israel) at 37°C , 95% air and 5% CO_2 . If necessary, cells were subcultured in 75 cm^2 tissue culture flasks after the first passage until confluence. For treatments, cells were subcultured into 25 cm^2 tissue culture flasks (second or third passage), and the cell culture medium was changed to the treatment medium at 80% confluence.

To examine the effect of estrogen and progesterone on DNMTs mRNA levels in ESC, cells were incubated with treatment medium (phenol red-free DMEM supplemented with glutamine, antibiotics and 2% stripped FCS) containing a combination of estradiol (10^{-8} M) (Sigma) and medroxyprogesterone acetate (MPA, 10^{-6} M) (Sigma) for 17 days at 37°C , in 95% air and 5% CO_2 . The concentrations of estradiol and MPA, and the period of incubation were based on our previous reports (Sugino *et al.*, 2000, 2002d). The medium was changed every other day. Decidualization was

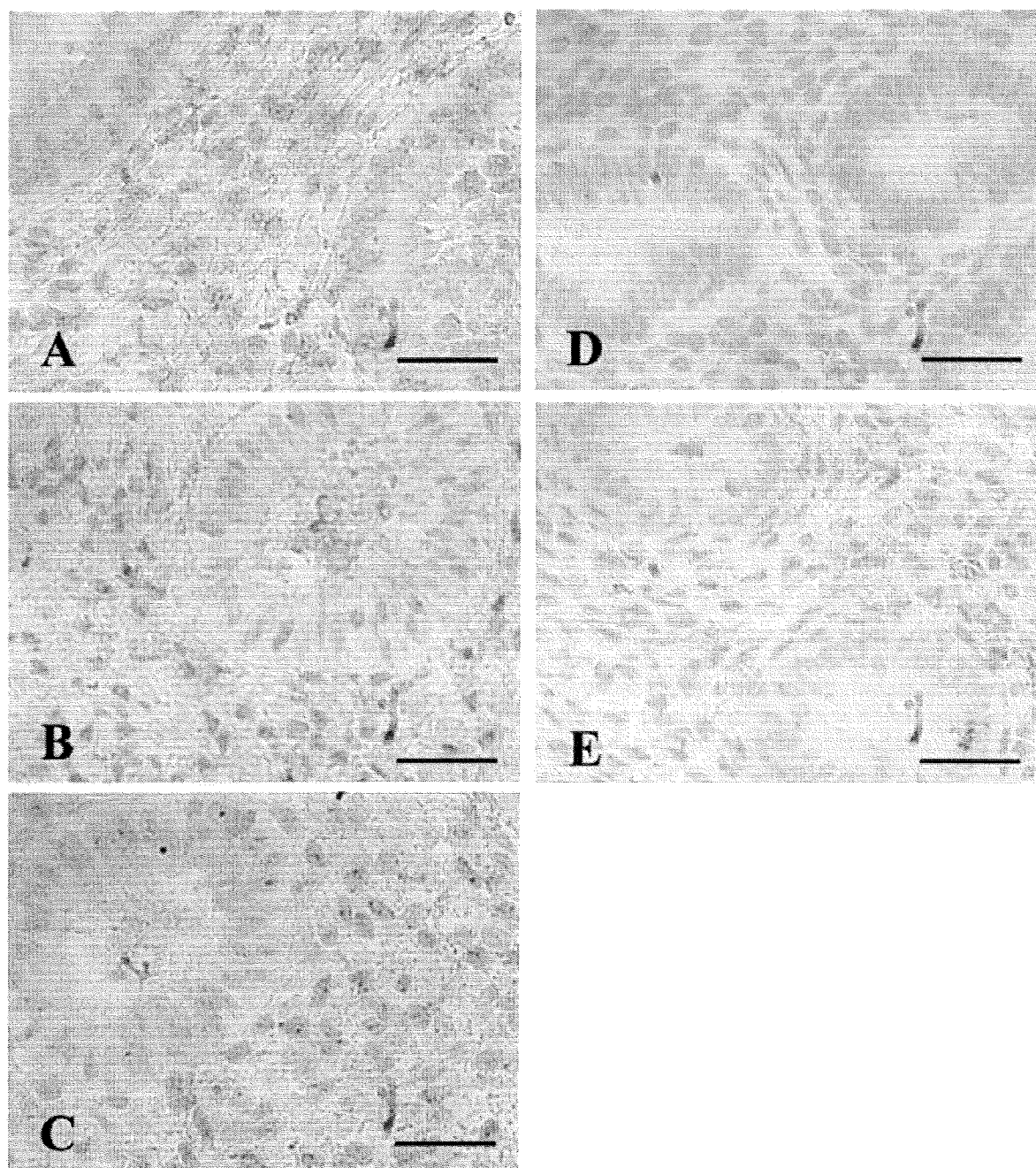


Figure 1 Immunohistochemical staining for DNMT1, DNMT3a and DNMT3b in the human endometrium. Immunohistochemical staining was performed on tissue samples from the proliferative phase, early secretory phase, mid-secretory phase and late secretory phase. Tissue samples were taken from three different patients in each phase. Representative results from the late proliferative phase are shown. Nuclei of both epithelial cells and stromal cells immunostained for DNMT1 (**A**), DNMT3a (**B**) and DNMT3b (**C**). No immunoreactivity was observed in the control sections incubated with normal mouse serum (**D**) and normal rabbit serum (**E**). Bar = 50 μ m.

confirmed by morphology and mRNA expression of insulin-like growth factor binding protein-1 (IGFBP-1), which is a specific marker of decidualization (Giudice *et al.*, 1992; Sugino *et al.*, 2000). Total RNA was isolated from cultured cells, and RT-PCR for DNMTs was performed as described below, with a duplicate PCR for each culture.

Real-time RT-PCR

Total RNA was extracted from endometrial tissues and cultured cells using Isogen (Wako, Osaka, Japan), and real time RT-PCR was performed as reported previously (Asada *et al.*, 2008). RT reactions were performed with ExScript RT reagent kit (TAKARA, Kyoto, Japan) according to the

manufacturer's protocol. Briefly, 2 µg of total RNA was incubated with 4 µl of 5 × ExCripT buffer, 1 µl of dNTP mixture (10 mM each), 1 µl of Random primers (50 µM), 0.5 µl of ExCripT reverse transcriptase (200 U/µl) and 0.5 µl of RNase inhibitor (40 U/µl) in 20 µl of reaction mixture at 42 °C for 15 min, after which the reverse transcriptase was inactivated by heating the samples at 95 °C for 2 min. The complementary DNA (cDNA) was immediately used for PCR. All PCRs were performed using SYBR Premix Ex Taq (TAKARA) and a LightCycler (Roche Applied Science, Basel, Switzerland). Briefly, 2 µl of aliquots containing cDNA were amplified in a total volume of 20 µl containing 4 µl of a 5 × SYBR Premix Ex Taq and 0.2 µM each primer. For internal controls, TATA box binding protein (TBP) cDNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also amplified. According to the previous report (Girault et al., 2003), the following primers were used. DNMT1 (forward, 5'-TACCTGGACGACCCTGACCTC-3', reverse, 5'-CGTTGCATCAAAGATGGACA-3') (product size: 103 bp); DNMT3a (forward, 5'-TATTGATGAGCGCACAAGAGAGC-3', reverse, 5'-GGGTGTTCCAGGTAACATTGAG-3') (111 bp); DNMT3b (forward, 5'-GGCAAGTTCTCCGAGGCTCTCTG-3', reverse, 5'-TGGTACATGGCTTTTCGATAGGA-3') (113 bp); TBP (forward, 5'-TGCACAGGAGCCAAGAGTGA-3', reverse, 5'-CACATCACAGCTCCCAACCA-3') (132 bp); IGFBP-1 (forward, 5'-CGAAGGCTCTCCATGTACCA-3', reverse, 5'-TGCTCTCTGTGCCTTGGCTAAAC-3') (98 bp) and GAPDH (forward, 5'-AGGTGAAGGTCGGAGTCA-3', reverse, 5'-GGTCATTGATGGCAACAA-3') (99 bp). All samples were run in duplicate. For appropriate negative controls, the RNA template was replaced with nuclease-free water in each run. Melting curves of the products were obtained after cycling by a stepwise increase of temperature from 55 to 95 °C. At the end of 40 cycles, reaction products were separated electrophoretically on an agarose gel and stained with ethidium bromide for visual confirmation of the PCR products.

Statistical analyses

Statistical analysis was carried out using the Statistical Package for the Social Sciences for windows 13.0. To evaluate whether tissue mRNA levels significantly vary during the menstrual cycle, the Tukey honest significant difference test was used. For ESC cultures, differences in mRNA levels were determined using Duncan's new multiple range test. $P < 0.05$ was considered to be significant.

Results

Nuclei of both epithelial cells and stromal cells in tissue sections immunostained for DNMT1, DNMT3a and DNMT3b during each phase of the menstrual cycle. Representative results from the late proliferative phase are shown in Fig. 1. The staining intensities did not vary among the menstrual phases.

Changes in DNMT1, DNMT3a and DNMT3b mRNA levels in the endometrial tissue are shown in Fig. 2. DNMT1 mRNA levels were significantly lower in the mid-secretory phase than in the other menstrual phases (Fig. 2A). DNMT3a mRNA levels were significantly lower in the secretory phase than in the proliferative phase (Fig. 2B), being lowest in the mid-secretory phase (Fig. 2B). The pattern of change in the DNMT3b mRNA levels was similar to that for DNMT3a, but the changes were not statistically significant (Fig. 2C).

Since mRNA levels of DNMT1, DNMT3a and DNMT3b in the endometrium were lower in the mid-secretory phase than in the proliferative phase, we examined whether DNMT mRNA expression is influenced by progesterone and estrogen. We therefore focused on

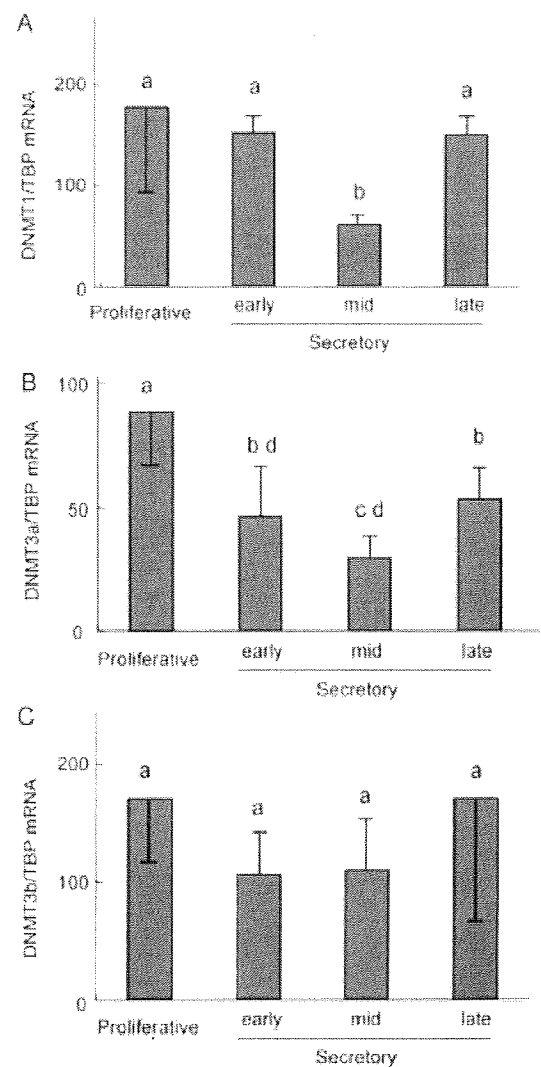


Figure 2 Changes in mRNA levels of DNMT1 (A), DNMT3a (B) and DNMT3b (C) in the human endometrium during the menstrual cycle. Total RNA was isolated from endometrial tissues and subjected to real-time RT-PCR. Endometrial tissues were obtained from the proliferative phase ($n = 14$), early secretory phase ($n = 10$), mid-secretory phase ($n = 11$) and late secretory phase ($n = 7$). Relative mRNA expression normalized to TBP (internal control) was calculated. Values are mean \pm SD. Different letters indicate significant differences between groups ($P < 0.01$ in A, $P < 0.05$ in B).

ESC, which differentiate to decidualized stromal cells under the influence of progesterone and estrogen during the secretory phase. In order to induce decidualization *in vitro*, ESC were treated with MPA and estradiol for 17 days. As shown in Fig. 3A, mRNA expression of IGFBP-1, a specific marker of decidualization, was clearly induced by MPA and estradiol for 17 days. DNMT3a and DNMT3b mRNA levels were gradually decreased by MPA + estradiol and were significantly lower in the MPA + estradiol group than in the control group on days 8 and 17 after treatment (Fig. 3C and D). However, DNMT1 mRNA levels did not change during the treatment (Fig. 3B).

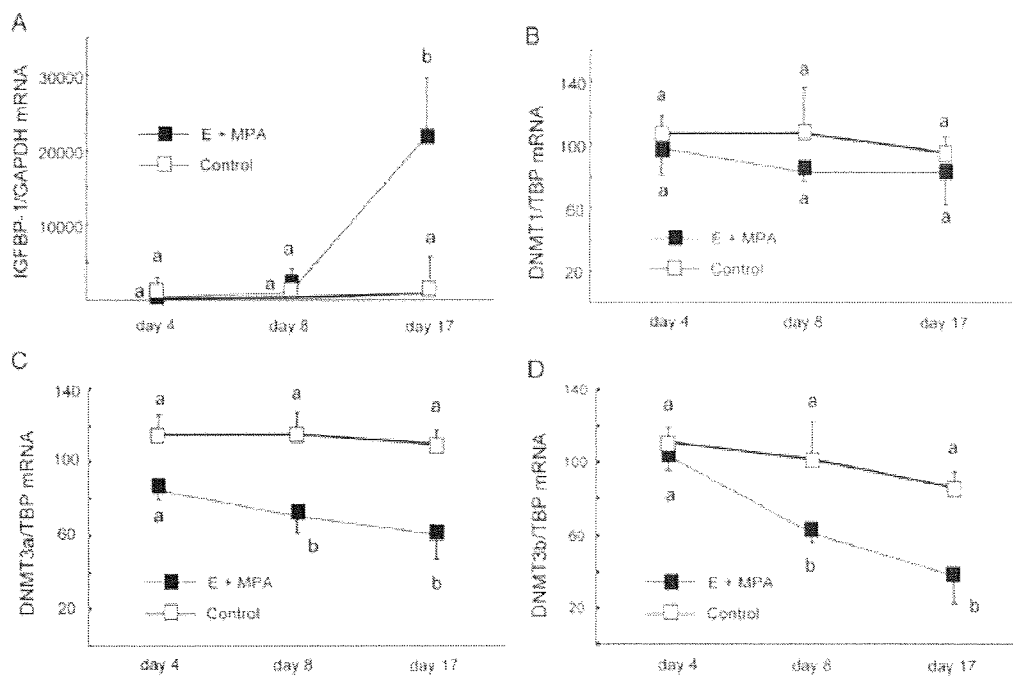


Figure 3 Effects of progesterone and estrogen on mRNA expression of DNMT1 (**B**), DNMT3a (**C**) and DNMT3b (**D**) in cultured ESC. Tissues were obtained from three individuals, and the cells from an individual were cultured in triplicate. Cells were treated with estradiol (E , 10^{-8} M) and MPA (10^{-6} M) for 17 days to induce decidualization *in vitro*. Decidualization was confirmed by mRNA expression of IGFBP-1, which is a specific marker of decidualization (**A**). Total RNA was isolated from cultured cells, and real-time RT-PCR for DNMTs or IGFBP-1 was performed and relative mRNA expression was calculated as described in Materials and Methods section. Values are mean \pm SEM of three different cultures. Different letters indicate significant differences between groups ($P < 0.05$ in A and D, $P < 0.01$ in C).

Discussion

The present study showed changes in the level of DNMT mRNAs in the human endometrium during the menstrual cycle. DNMT3a mRNA levels were significantly lower in the secretory phase than in the proliferative phase, being lowest in the mid-secretory phase. The pattern of change in DNMT3b mRNA levels was similar to that for DNMT3a. Furthermore, we showed that DNMT3a and DNMT3b mRNA level in ESC was down-regulated by MPA and estrogen. DNMT3a and DNMT3b are responsible for *de novo* CpG methylation (Hsieh, 1999). CpG methylation of the promoter region inactivates gene expression (Nan et al., 1998; Ushijima and Okochi-Takada, 2005). These findings lead us to speculate that the down-regulation of DNMT3a and DNMT3b mRNAs may be associated with expression of the genes that are induced during decidualization. In fact, a great number of genes are up-regulated and some genes are newly expressed in the human endometrium undergoing decidualization (Popovici et al., 2000; Kao et al., 2002; Riesewijk et al., 2003; Ace and Okulicz, 2004; Mirkin et al., 2005). Further studies are needed to find out which genes are regulated by DNA methylation during decidualization.

In the present study, DNMT1 mRNA levels in the endometrial tissue were significantly lower in the mid-secretory than in the proliferative phase, whereas DNMT1 mRNA levels were not affected by

MPA and estradiol in ESC undergoing decidualization. Therefore, the low levels of DNMT1 in the mid-secretory phase endometrium may reflect the levels in the endometrial epithelium rather than the levels in the ESC. DNMT1 is responsible for accurately replicating genomic DNA methylation patterns to maintain genome stability during cell division (Liu et al., 1998). Endometrial epithelial cells do not proliferate during the mid-secretory phase, which seems to be compatible with the decreased DNMT1 expression during the mid-secretory phase.

There seems to be a discrepancy in this study between mRNA and protein levels for DNMT, and this may be due to the different sensitivities of RT-PCR and immunohistochemistry.

Little information is available regarding the regulation of DNMT expression. Interestingly, the present study showed that DNMT3a and DNMT3b are under the regulation of female sex steroid hormones, suggesting that DNA methylation may be influenced by female sex steroid hormones. It has been reported that DNA methylation status can be altered by a variety of factors including steroids and vitamins (Shiota, 2004). On the other hand, DNA methylation affects estrogen receptors in endometria, mammary glands and myometrium (Lapidus et al., 1996; Iwase et al., 1999; Giacinti et al., 2006; Asada et al., 2008). These findings suggest a close relationship between DNA methylation and female sex steroid hormones. However, further study is needed to clarify the molecular mechanisms of regulation of DNMTs expression.

The phase-specific and transient changes in the DNMT mRNAs during the menstrual cycle may suggest that DNA methylation status is changeable during the menstrual cycle, which may lead to changes in transcription levels of some genes. This is supported by recent reports that DNMTs are involved in both methylation and demethylation of CpG dinucleotides in human cells with cyclical changes in DNA methylation status (Kangaspeska et al., 2008; Metivier et al., 2008).

This study showed that DNMT mRNA levels change in the human endometrium during the menstrual cycle and that DNMT3a and DNMT3b mRNAs in ESC can be regulated by female sex steroid hormones. These results suggest that DNMTs have regulatory functions on gene expression in the human endometrium. Further studies are needed to show a potential role of epigenetic regulation in gene expression that is associated with decidualization.

Authors contribution

Y.Y.: conception and design, acquisition of data, analysis of data and drafting the article; H.A., L.L., I.T., R.M., K.T., T.T., A.M. and H.T.: acquisition of data and N.S.: conception and design, interpretation of data, drafting the article and final approval.

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Epigenetics for Biomedical Sciences

Mitsuko Hirosawa-Takamori, PhD, Hui Wen Lim, PhD,
Shintaro Yagi, PhD, and Kunio Shiota, DVM, PhD

Abstract: Epigenetics is fundamental to the development and maintenance of cellular phenotypes. Epigenetic status is inherited by the next cell generation and is changeable during the process of cellular differentiation. DNA methylation, in association with histone modifications and chromatin condensation, is a major epigenetic event that causes gene silencing. Every cell type or tissue has a unique DNA methylation profile comprising at least thousands of tissue-dependent differentially methylated regions (T-DMRs), suggesting that the epigenetic changes at T-DMRs underlie cellular differentiation. Genome-wide epigenetic information is called the epigenome. Disruption of the normal epigenome produces aberrant cells with prolonged abnormal phenotypes. Epimutation may be more common than previously thought considering there are numerous T-DMRs in the mammalian genome. Mutation and epimutation are inherited by the next cell generation, and both continue throughout life. However, epimutation is reversible, and drug treatment could potentially reverse the epigenetic status. Analysis of the epigenome will provide insight into novel advances in diagnosis and treatment of, as well as drug development for, complex chronic diseases.

Key Words: epigenetics, DNA methylation, epigenome, tissue-dependent differentially methylated regions (T-DMRs), human diseases

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Epigenetics has been defined as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence.”¹ Recently, epigenetics has been more broadly defined as “the study of processes that produce a heritable phenotype that does not strictly depend on DNA sequence.”²

Most human diseases are related in some way to loss or gain of gene function. Indeed, it has been established that >8000 human diseases are caused by defects in single genes.³ In addition, more complex diseases with chronic symptoms including diabetes,⁴ hypertension,⁵ and corneal dystrophies⁶ are thought to be related to multiple gene mutations. To study the genetic component of many human

diseases, large sets of human single-nucleotide polymorphisms (SNPs) have been identified and mapped to referenced human genome sequences.⁷ For human health, the importance of mutation research is well recognized because pathological changes caused by mutations may continue throughout life.

“Heritable changes in gene function without changes in DNA sequence” are a fundamental phenomenon of mammalian development. A single fertilized egg gives rise to a complex multicellular organism consisting of various differentiated cell types. Based on a variety of physiological and morphological criteria, there are >200 differentiated cell types in mammals. In general, cellular differentiation occurs without changes in DNA sequence. Cell phenotypes may be maintained even after mitosis, when the cell is equipped with epigenetic mechanisms that enable the inheritance of gene function.

Epigenetic mechanisms regulate DNA accessibility for extended periods as described in this review. Therefore, epimutation should be recognized as another critical cause of general and chronic diseases because the resulting pathological conditions are theoretically continued for extended periods, even throughout a human life.

EPIGENETIC MECHANISMS OF DNA METHYLATION AND HISTONE MODIFICATIONS

Methylation of cytosine residues in DNA, a unique DNA modification in vertebrate genomes, is the most studied among epigenetic inheritance mechanisms. DNA methylation occurs at the 5'-position of cytosine in a 5'-CG-3' dinucleotide (C paired G; CpG).^{8,9} Methylcytosine is the only chemical modification of mammalian genomic DNA. The status of methylated or unmethylated CpGs is maintained after DNA replication; therefore, the DNA methylation signal is heritable throughout future cell generations, indicating that DNA methylation is a vehicle for transferring epigenetic information.¹⁰

Another major epigenetic mechanism is modification of histone proteins. To date, >60 different residues on core histones (H2A, H2B, H3, and H4) have been reported as modified. Modifications include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, adenosine diphosphate ribosylation, deimination, and proline isomerization.¹¹

In the nucleus, DNA is wrapped around clusters of globular histone proteins to form nucleosomes that are organized into chromatin. Changes to chromatin structure influence gene expression: genes are inactivated (switched off) when chromatin is condensed (silent) and expressed (switched on) when chromatin is open.^{12–14} These dynamic chromatin

From the Laboratory of Cellular Biochemistry, Animal Resource Sciences/Veterinary Medical Sciences, Graduate School of Agriculture and Life Sciences, University of Tokyo, Tokyo, Japan.

Reprints: Kunio Shiota, DVM, PhD, Laboratory of Cellular Biochemistry, Animal Resource Sciences/Veterinary Medical Sciences, Graduate School of Agriculture and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan (e-mail: ashiota@mail.ace.u-tokyo.ac.jp).

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states are controlled by reversible epigenetic patterns of DNA methylation and histone modification, which play crucial roles in gene silencing and stabilizing chromosomal structure¹²⁻¹⁴—as shown in Fig. 1. Epigenetic mechanisms underlie various biological phenomena that include differentiation,¹⁵ development,^{16,17} X-inactivation,¹⁸ and imprinting.^{19,20}

DYNAMIC CHANGES IN DNA METHYLATION

The maternal genome is demethylated gradually, whereas the paternal genome undergoes rapid DNA demethylation immediately after fertilization.²¹ However, change in the methylation status of bulk DNA is not always parallel with changes in gene coding areas.

Human and mouse genomes consist of a 3×10^9 base pairs per haploid genome, and a large amount of the genome is composed of nongenic repetitive elements, including interspersed repeats and satellites.²²⁻²⁴ Approximately 60%–90% of CpGs are methylated in the mammalian genome.^{9,25,26} Therefore, global demethylation of the maternal or paternal genome immediately after fertilization²¹ mainly reflects changes in methylation levels in these nongenic loci. Analysis of genic area DNA methylation reveals that changes occur by de novo methylation and demethylation after fertilization, depending on the loci,²⁷ and the epigenetic status of gene coding areas shows more dynamic changes than nongenic regions.

CPG ISLANDS WITH TISSUE-DEPENDENT DIFFERENTIALLY METHYLATED REGIONS

In mammals, CpGs exist in relatively low numbers compared with estimates based on average base pair composition. CpGs are unevenly distributed throughout the

mammalian genome and form clusters near gene coding regions. These CpG-rich regions are called CpG islands.^{28,29} Gardiner-Garden and Frommer²⁹ originally defined CpG islands as regions >200 base pairs with high GC content and an observed/expected ratio for the occurrence of CpG >0.6 . A slight modification of the definition has been proposed to exclude small repetitive sequences.³⁰ CpG islands usually stretch approximately 1–2 kb and are located most often near promoters and early exons of genes. Therefore, CpG islands have been used as landmarks to find gene coding regions in the genome.^{28,29,31} The CpG islands have long been believed unmethylated in normal tissues.^{26,32-34} However, it became evident that there are many CpG island-associated genes that are repressed by DNA methylation.

The sphingosine kinase 1 (*Sphk1*) gene, consisting of 6 messenger RNA subtypes (*Sphk1a-f*), is an example of a CpG island-associated gene repressed by DNA methylation.³⁵ Sphingosine kinase 1 catalyzes production of sphingosine 1-phosphate, an intracellular signal messenger that plays critical roles in signal transduction, cell growth, and differentiation.³⁶ In the upstream region of the *Sphk1* gene, there is a CpG island with a tissue-dependent differentially methylated region (T-DMR). Methylation status of the T-DMR is highly correlated with expression of *Sphk1a*. When *Sphk1a* is expressed in the adult rat brain, T-DMR is hypomethylated, whereas it is hypermethylated and silenced in other tissues. Several other CpG island-associated genes such as those encoding e-cadherin,³⁷ endothelin receptor B,³⁸ and proopiomelanocortin³⁹ are reported to have T-DMRs with methylation states that are correlated with gene expression. Recent studies indicate that there are numerous CpG island-associated genes with T-DMRs.⁴⁰

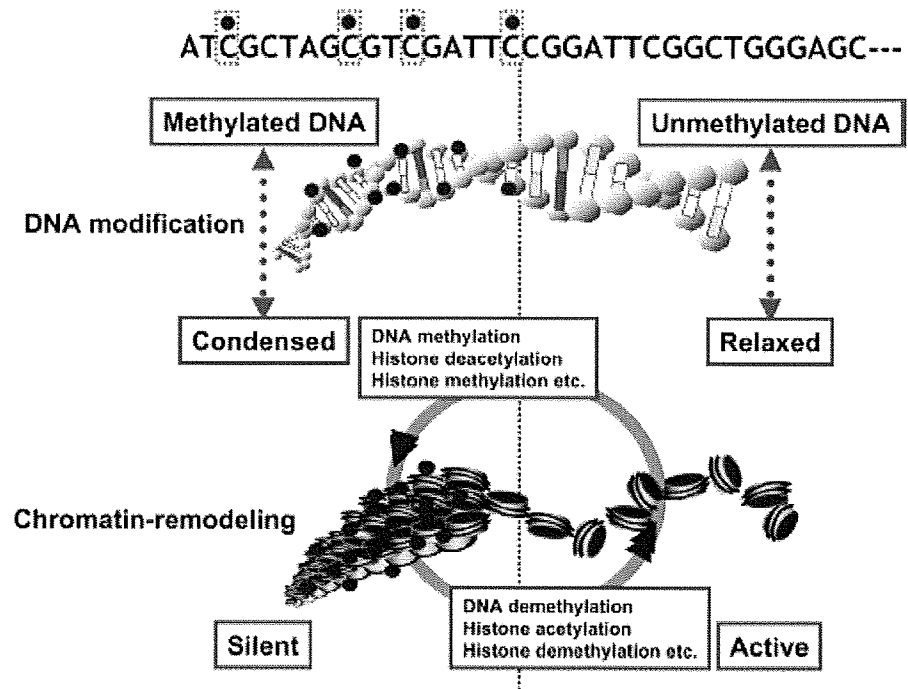


FIGURE 1. Epigenetic regulation by DNA methylation links to chromatin remodeling. Methylation of CpGs occurs in the regulatory region of genes. Methylation of DNA and chromatin structure are coordinated through various modifications of core histones, including acetylation and methylation. In general, DNA methylation and chromatin condensation are associated with gene silencing.