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ORIGINAL RESEARCH

Aberrant DNA methylation status in human uterine leiomyoma

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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 (Chiaffarino *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 [α-^{32}P]dCTP and [α-^{32}P]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, XAR5, 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 (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.

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.

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 (Hattori *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 NH_4OAc (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 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) 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′-CCTCTCCGCCTCCCGATGG-3′		Methylation-sensitive
	R:5′-TGATGTGTGTTTGGTGAGCAAGG-3′		quantitative real-time PCR
GS20656	F:5′-GGGGTGGTTAATTTTAGAGATGATT-3′		Sodium bisulfite sequencing
	R:5′-TTACTCTCCAAAACCAAATACCAAA-3′		
GS20656	F:5′-CAGCTGGTCACGTCCTCAC-3′	133	Real-time RT–PCR (primer set I)
	R:5′-CCAAAGGAGAGGCACAAAAG-3′		
GS20656	F:5′-CAGCTGGTCACGTCCTCAC-3′	250	RT–PCR (primer set II)
	R:5′-CAACAATTGCCTCACAATCG-3′		
DNMT1	F:5′-TACCTGGACGACCCTGACCTC-3′	103	Real-time RT–PCR
	R:5′-CGTTGGCATCAAAGATGGACA-3′		
DNMT3a	F:5′-TATTGATGAGCGCACAAGAGAGC-3′	111	Real-time RT–PCR
	R:5′-GGGTGTTCCAGGGTAACATTGAG-3′		
DNMT3b	F:5′-GGCAAGTTCTCCGAGGTCTCTG-3′	113	Real-time RT–PCR
	R:5′-TGGTACATGGCTTTTCGATAGGA-3′		
TBP	F:5′-TGCACAGGAGCCAAGAGTGAA-3′	132	Real-time RT–PCR
	R:5′-CACATCACAGCTCCCCACCA-3′		
GAPDH	F:5′-AGGTGAAGGTCGGAGTCA-3′	99	Real-time RT–PCR
	R:5′-GGTCATTGATGGCAACAA-3′		

F, forward primer; R, reverse primer.

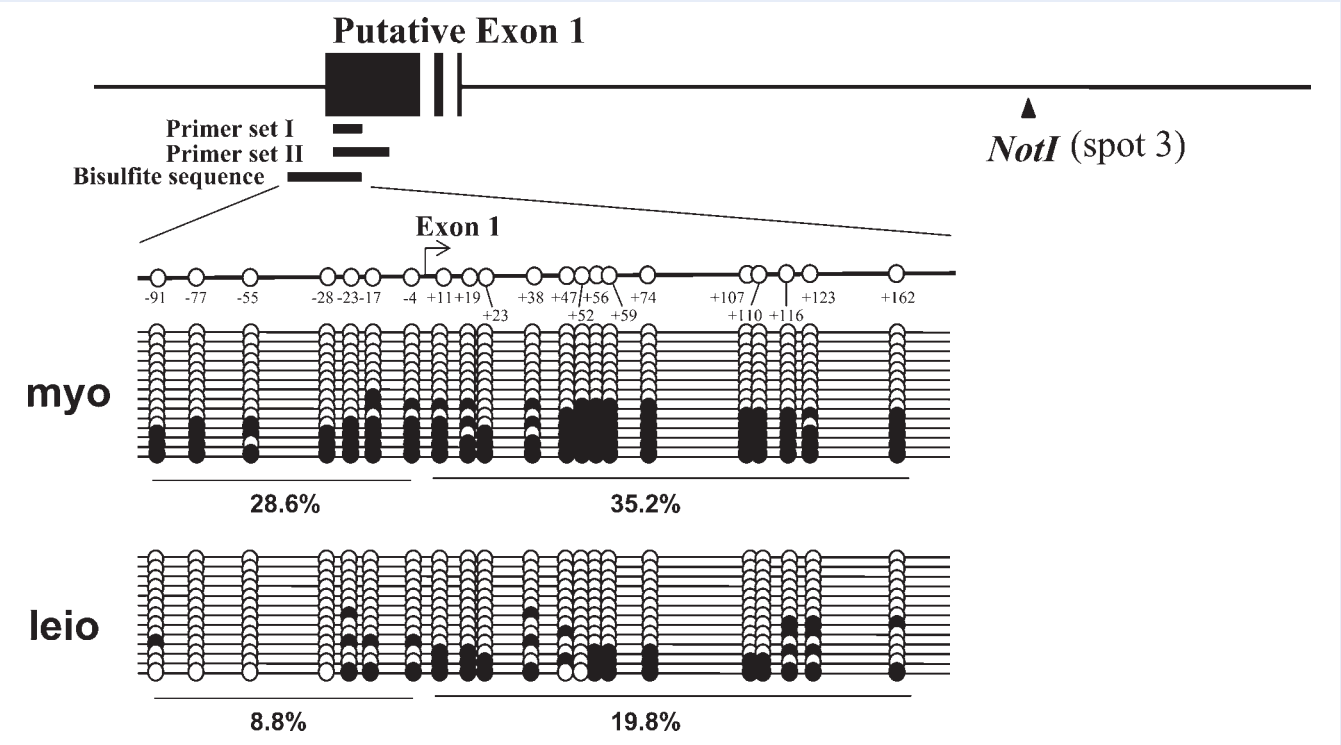

Figure 1 DNA methylation status of the 5′-flanking region of GS20656 in normal myometrium and uterine leiomyoma. The region of the first exon is shown as a thick black bar. The boundary between the first exon and the first intron was blurred. The arrowhead indicates NotI site of the spot #3 in the RLGS 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 ± 3.0%) was significantly lower than that in the normal myometrium (52.2 ± 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).

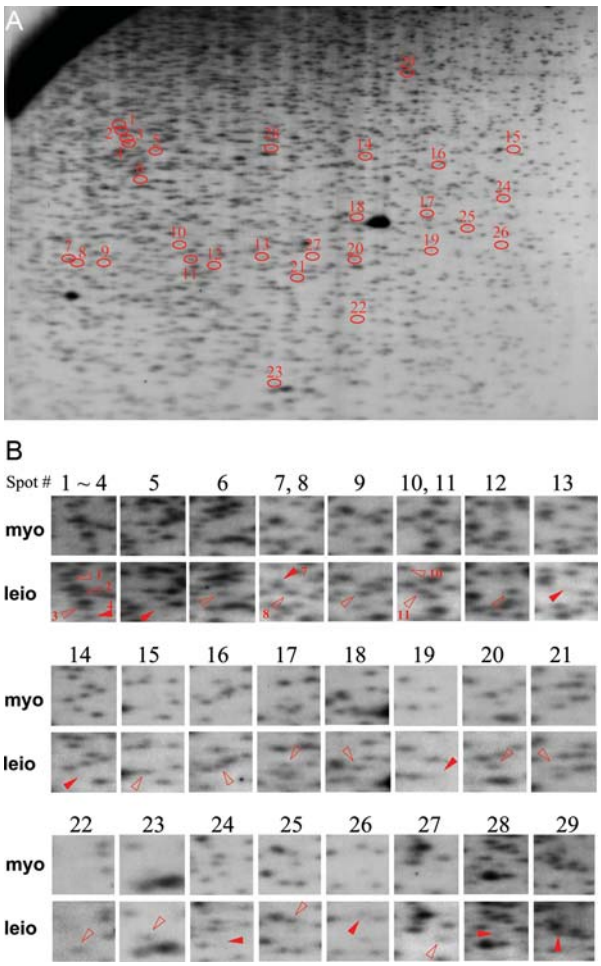

Figure 2 Genome-wide DNA methylation analysis of uterine leiomyoma and the corresponding normal myometrium. (**A**) Representative RLGS profile of a normal myometrium. Numbered circles indicate the 29 spots in which the intensity of spots was either higher or lower than the corresponding spots in the 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,

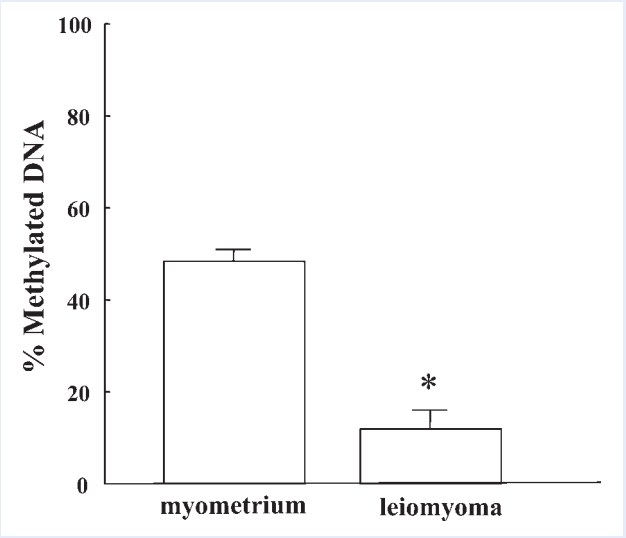

Figure 3 Percentage methylated DNA at the spot #3 locus in the normal myometrium and uterine leiomyoma. DNA methylation levels were determined by methylation-sensitive quantitative real-time RT–PCR as described in the Materials and Methods section. Values are mean + SEM of three different experiments. *$P < 0.05$ 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 on 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

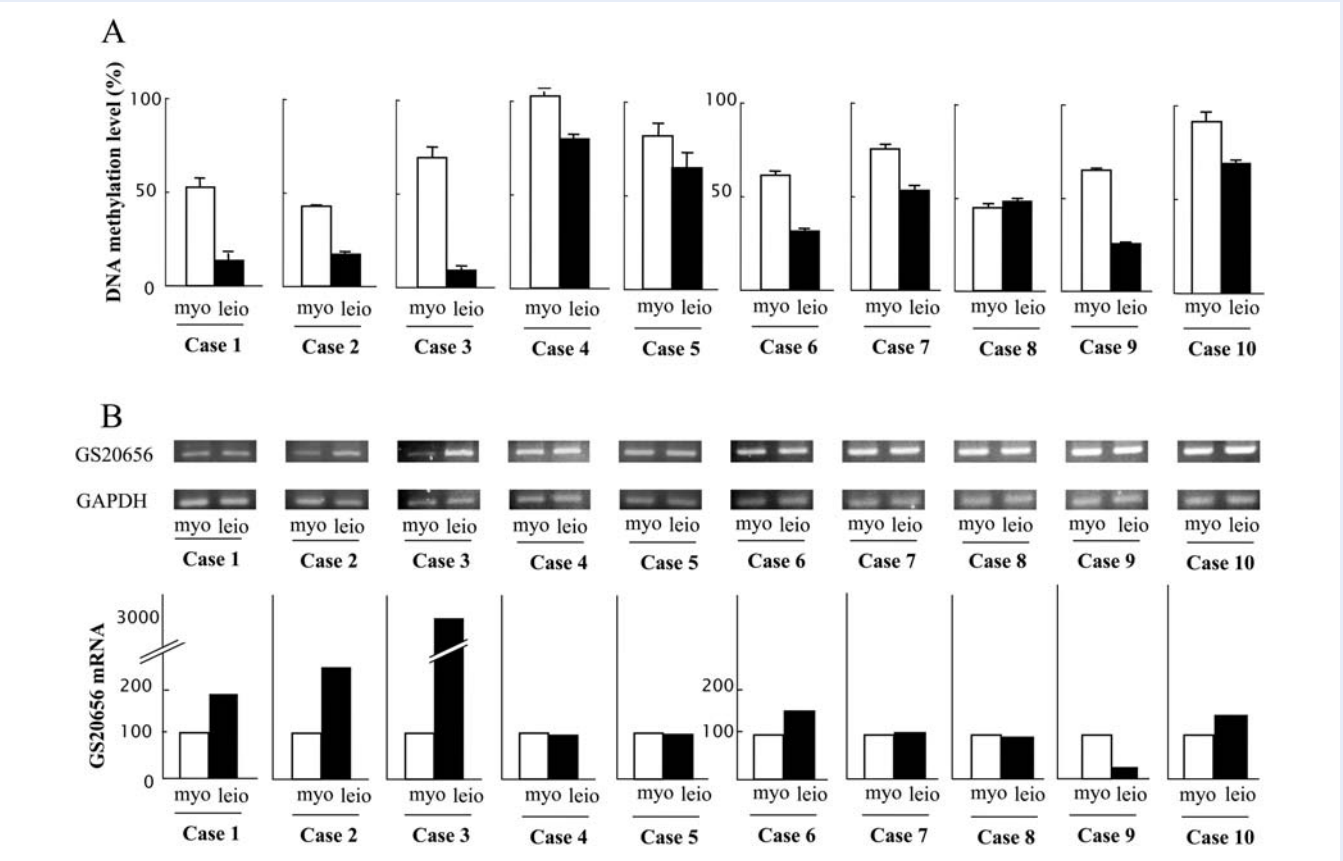

Figure 4 DNA methylation levels of the first exon of GS20656 and mRNA levels of GS20656. Methylation status of the first exon of GS20656 and mRNA expression of GS20656 were examined for 10 paired samples with leiomyoma (leio) and myometrium (myo). (**A**) DNA methylation levels were evaluated by methylation-sensitive quantitative real-time PCR, and expressed as %methylated DNA. Values are mean ± 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

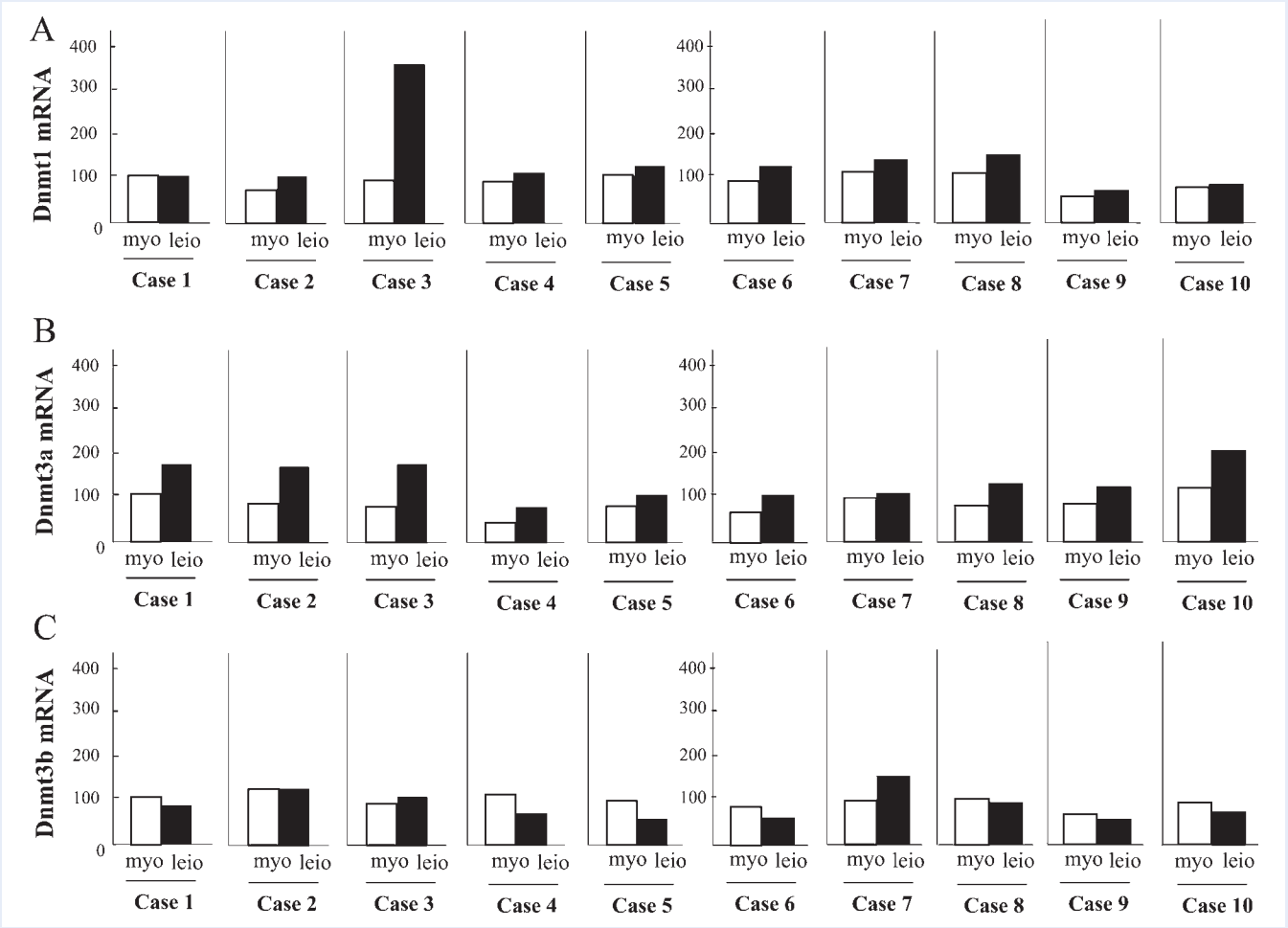

Figure 5 mRNA expression of DNMT in uterine leiomyoma (leio) and myometrium (myo). mRNA levels of DNMT1 (**A**), DNMT3a (**B**) and DNMT3b (**C**) were analyzed by real-time RT–PCR on the same 10 paired samples as shown in Fig. 4.

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.
[a]$P < 0.01$ versus myometrium.
[b]$P < 0.05$ 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.

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