Does Aberrant DNA Methylation Occur in Human Uterine Leiomyomas? An Attempt of Genome-Wide Screening by MS-RDA

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Objective : Uterine leiomyoma are very common benign tumors in women of reproductive age. However, the molecular mechanisms of cause and development of these tumors are poorly understood. This study attempts to examine whether or not aberrant DNA methylation occurred in these tumors.
Methods : We carried out a genome-wide screen for aberrant DNA methylation, adopting methylation-sensitive-representational difference analysis (MS-RDA) using normal adjacent myometria as tester and myoma tissue driver.
Conclusion : A total of 192 clones identified by MS-RDA were sequenced, 27 DNA fragments derived from CpG islands (CGIs) were isolated, and seven of them were from CGI in the 5' regions of known genes, which include CHARC1, FAM44B, FLJ33655, HSUP, MLLT3, SLC16A1, and ZNF96. Then, methylation statuses of those CGIs were analyzed by methylation-specific polymerase chain reaction using 5 primary samples of human uterine leiomyoma. Aberrant DNA methylation did not observed in 7 genes in 5 human uterine leiomyoma eventually. This study is insufficient to identify aberrant DNA methylation occurring in the human uterine leiomyoma, a large population of primary samples and more attempts, such as the use of cell lines or primary monolayer cultures established from tissue samples, are warranted to clarify this issue.

Key words: MS-RDA, uterine leiomyoma, CpG island, DNA methylation

INTRODUCTION

Uterine leiomyomas are the most common benign gynecological tumor and occur in 25% of women of reproductive age [1]. Various clinical problems such as pelvic pain, abnormal uterine bleeding, urinary frequency, infertility, and recurrent pregnancy loss are attributed to this disease [2, 3]. Though remarkably literature about their epidemiology, cytogenetics, molecular genetics and hormonal aspects are published [4–7], the molecular mechanisms of cause of these tumors remain unclear. Previous studies suggest that uterine leiomyomas are monoclonal tumors that origin from smooth muscle cells [8]. Cytogenetic analyses showed that some chromosome are changed, such as trisomy 12, translocation between chromosome 12 and 14, deletions of chromosomes 3 and 7, rearrangement of short arm of chromosome 6 and of the long arm of chromosome 10 [4, 9]. In addition, increasing evidence has demonstrated that sex steroid hormones and growth factors play central roles in leiomyoma development and growth [7, 10–12]. Since the steroid hormone levels in women with leiomyoma are similar to those in normal women, sex steroids are not considered to be the sole modulators of tumorigenesis [13]. Therefore, the underling molecular mechanisms for tumorigenesis remain to be elucidated.

Alterations of DNA-methylation patterns, as an epigenetic modulation, both the regional hypermethylation and the global hypomethylation in genomic DNA, are deeply involved in many human tumor types [14]. DNA methylation defined as methylation of the C5 position of cytosine/guanine pairs (CpG) in DNA, and has been observed in CG-rich region, called CpG islands (CGIs), frequently located in a promoter. These CGIs are normally kept free of methylation in promoter regions for proteins binding and initiating gene transcription. However, methylated CGIs lead to stable heritable transcriptional silencing of tumor-suppressor genes and have been considered as common features in human carcinomas [15]. Moreover, aberrant DNA methylation was also shown to be present in noncancerous mucosae of ulcerative colitis and H. pylori-infected gastric mucosae in previous studies [16, 17], a role of chronic inflammation in methylation induction was proposed.

On the other hand, global hypomethylation frequently targeted repetitive sequences [18] have been demonstrated to contribute to tumorigenesis and progression through effects on chromosomal stability [19]. Li *et al*. demonstrated that global DNA hypomethylation and differential expressions of different DNA

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methyltransferases (DNMT1, 3A and 3B) in uterine leiomyoma tissue as compared with the adjacent myometria, suggesting a potential mechanism of epigenetic modulation in the development of this tumor [20]. Moreover, aberrant promoter methylation of cancer-related genes has been detected in leiomyosarcomas, such as ERa (80%), DAP kinase (54%), RASSF1A (39%), p16INK4a (22%–25%), and MLH1 (6%) [21–23]. Therefore, it is of interesting to investigate whether aberrant DNA methylation is involved in the development of the benign tumor of human leiomyoma.

For this purpose, we adopted a genome-wide screening for differences in DNA methylation, methylation-sensitive-representational difference analysis (MS-RDA) [24–26]. MS-RDA is a power technique to isolate differentially methylated DNA fragments between two genomes. In MS-RDA, genomic DNA is first digested with a methylation-sensitive restriction enzyme that has a four-base or six-base recognition sequence, such as HpaII, SacII, or NarI. And genomic DNA can be only cut at unmethylated recognition sites. Then an adaptor is ligated to the restricted site. The ligation products are then PCR amplified using the adaptor as primer. This procedure produces a DNA fragments library derived from unmethylated CpG-rich regions of the genome, while miss to produce a library derived from methylated CpG-rich of the genome because that methylation-sensitive restriction enzyme can not cut methylated recognition sites. Therefore, the two different DNA fragment libraries can be isolated by RDA. Adopting MS-RDA technology, it has been successfully to identify various aberrant methylation and silenced genes in human lung cancers [27], stomach cancers [28], pancreatic cancers [29], breast cancers [30, 31], neuroblastomas [32] and ovarian cancers [33].

MATERIALS AND METHODS

Tissue samples and DNA extraction

Leiomyomas were obtained from five patients undergoing surgical treatment for this disease at Tokai University Hospital. Informed consent was obtained from all patients. As normal control, adjacent uterine myometria of 0.5–1.0 cm was collected from the same patient. Tissue samples were stored at -80℃ until used. DNA was extracted by a standard phenol/chloroform and ethanol precipitation procedure.

MS-RDA and database search

For MS-RDA [24, 31], genomic DNA of myoma tissue and adjacent myometria from the identical patient (case 1) was prepared and digested with HpaII, which is a methylation sensitive restriction enzyme that prevented by the presence of a 5-methyl group at the internal C residue of its recognition sequence CCGG. The pooled DNA of myoma tissue was used as driver, and the pooled DNA of adjacent myometria was used as tester in this study. In briefly, R adaptor (RHpa24: 5'-AGCACTCTCCAGCCTCTCA-CCGCA-3'; RHpaII: 5'-CGGTCGGTGAG-3') was ligated to 1 μg of genomic DNA digested with HpaII (New England Biolabs, Beverly, MA, USA). Then the ligation product was amplified by 25 cycles of PCR with RHpa24 oligonucleotide in the presence of 1 M betaine (Sigma, St. Louis, MO, USA). PCR products (amplicon) of both tester and driver were digested with HpaII. J adaptor (JHpa24: 5'-ACCGACGTCGACTATCCATGA-AAC-3'; JHpa11: 5'-CGGTTTCATGG-3') was ligated only to the tester amplicon, and 200 ng of it was mixed with 40 μg of the driver amplicon. Then the mixture underwent heat denaturation and reannealing (competitive hybridization), and dsDNA with the J adaptor on both ends was selectively amplified (selective amplification) with JHpa24 oligonucleotide. The adaptor of the first competitive hybridization and selective amplification was switched to a new N adaptor (NHpa24: 5'-AGGCAACTGTGCTA-TCCGAGGGAC-3'; Nhpa11: 5'-CGGTCCCTCG-G-3'). Ligation product (40 ng) was mixed with 40 μg of driver amplicon, and the second cycle competitive hybridization and selective amplification were carried out. The final product was cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA), and total 192 clones were sequenced. Their genomic origins were examined by BLASTN software; chromosomal position and relative locations to CGIs were discovered at the GeneBank web site (http://www.ncbi.nlm.nih.gov).

Bisulfite modification and methylation-specific PCR

For sodium bisulfite modification, genomic DNA was restricted with BamHI (New England Biolabs Japan) and purified by phenol extraction. A total of 500 ng restricted DNA was denatured in 0.3 N NaOH at 37℃ for 15 min, then 15 cycles of denaturation was done at 95℃ for 30 sec and incubation at 50℃ for 15 min in 3.1 M NaHSO3 (pH = 5.0) and 0.5 mM hydroquinone. The product was desalted with the Wizard DNA Clean-Up System (Promega Corp., Madison, WI, USA), and desulfonated in 0.6 N NaOH at room temperature for 5 min, then ethanol precipitated and dissolved in 20 μl of TE buffer.

For methylation-specific PCR (MSP) [34], sodium-bisulfite-modified DNA was amplified with primer set specific to the methylated or unmethylated sequences. DNA from human ovarian surface epithelial (HOSE) was methylated in vitro using SssI-methylase (New England Biolabs), and used as a control for methylated DNA. MSP was done in a total volume of 20 μl, containing 1 μl modified template DNA, 1 μM of each primer, 0.2 mM deoxynucleotide triphosphates (Applied Biosystems), 2 μl 10 × PCR buffer with 15 mM Mg2+ (Applied Biosystems), and 0.5 unit AmpliTaq Gold (Applied Biosystems). MSP reactions were subjected to initial incubation at 95℃ for 10 min, followed by cycles of 95℃ for 30 seconds, and annealing at the appropriate temperature for 30 seconds and 72℃ for 30 seconds. To avoid confounding effects of low levels of unmodified DNA, the number of cycles of PCR used did not exceed 35 cycles. The CGIs analyzed are listed in (Table 1). Primer sequences and MSP conditions are detailed in Table 2. MSP products were separated on 2% agarose gels and visualized after ethidium bromide staining.

RESULTS

Isolation of putative aberrantly methylated CGIs by a genome-wide screening

Use of cell lines is commonly recommended for MS-

Table 1 Seven CpG Islands Methylation Analysis in Human Uterine Leiomyoma

Genes			Chromosomal location	Accession number	Map start position	CpG island		
Symbol	Description	Accession number				Length (bp)	%GC	ObsCpG/ ExpCpG*
CHARC1	chromatin accessibility complex 1	NM_017444	8q24.3	AC107375	56761†	2001	67.3	0.94
FAM44B	family with sequence similarity 44, member B	NM_138369	5q35.2	AC010339	95001†	1500	55.1	0.83
FLJ33655	hypothetical protein FLJ33655	NM_173641	1p34.3	AC104336	84609†	900	63.7	0.67
HSUP1	similar to RPE-spondin	XM_49776	20q13.13	AL049766	53976	2001	62.3	0.74
MLLT3	myeloid/lymphoid or mixed lineage-leukemia translocation to 3 homolog	NM_004529	9p22	AL354879	13221†	2001	61.4	0.81
SLC16A1	solute carrier family 16	NM_003051	1p12	AL158844	46763†	2000	62.3	0.80
ZNF96	zinc finger protein 96	NM_014724	6p22.2-p21.3	AC005678	61690†	1000	56.8	0.69

*ObsCpG/ExpCpG: observed CpG/expected CpG ratio; †: Reverse strand

Table 2 Primer Sequences and PCR Conditions for MSP

Genes	Methylation	Forward	Reverse	Annealing temperature (℃)	PCR production length (bp)
CHARC1	M	5'-TTTTCGGTTGTCGGTTTCGC-3'	5'-CCCGATCTACGCATACGCCG-3'	59	75
	U	5'-GATTTTTGGGAGTGGTGT-3'	5'-AAACTCCATAAAACCTCACA-3'	59	137
FAM44B	M	5'-TAATGTAAAGGTTAACGTTGAC-3'	5'-ATAAAAACGACGACGACG-3'	54	120
	U	5'-ATGTAAAGGTTAATGTTGAT-3'	5'-TAATAAAAACAACAACAACA-3'	54	120
FLJ33655	M	5'-GGTTGGTATTTCGCGGC-3'	5'-GAACTATCAATCCGACGACG-3'	59	149
	U	5'-ATTGGTTGGTATTTTGTGGT-3'	5'-ACCAAACTATCAATCCAACA-3'	57	155
HSUP1	M	5'-TATCGTTTATTTAGCGTTTC-3'	5'-AAATACTAAAAAAAACGACG-3'	54	132
	U	5'-TATTGTTTATTTAGTGTTTT-3'	5'-AAATACTAAAAAAAACAACA-3'	50	132
MLLT3	M	5'-GAGTTTTTTTTGGTTCGTTC-3'	5'-TAATTACGAAACATACGCCG-3'	56	122
	U	5'-AGTTTTTTTTGGTTTGTTT-3'	5'-TAATTACAAAACATACACCA-3'	53	121
SLC16A1	M	5'-CGTCGTTTAGTAGGGGCGTAGC-3'	5'-GTCTCTCCCGACCGCCG-3'	62	176
	U	5'-TGTTTAGTAGGGGTGTAGTGTGT-3'	5'-CATCTCTCCCAACCACCA-3'	58	174
ZNF96	M	5'-TTTTTTTTTTACGTAGACGC-3'	5'-ACCGAAAACGACCACG-3'	53	127
	U	5'-TTTTTTTTTATGTAGATGTGT-3'	5'-ACAAAAACCAAAAACA-3'	48	131

RDA. Due to few cell lines can be available for human leiomyomas, the primary samples were utilized. MS-RDA was performed with HpaII using a tissue sample from an identical patient. HapII is a methylation sensitive restriction endonuclease and prevented by the presence of a 5-methyl group at the internal C residue of its recognition sequence CCGG. In this study, adjacent myometria was used as the tester and myoma tissue was used as the driver to isolate DNA fragments specifically methylated in myoma tissue. Because methylation of promoter CGI leads to transcriptional silencing of their downstream genes [35, 36], we focus on the CGIs located at 5' region of genes. A total of 192 clones were obtained and sequenced, 62 of them were non-redundant. After BLAST search, 27 clones were found be derived from CGIs, and seven were flanked by CGIs in 5' region of genes (Table 1). Those cloned DNA fragments by MS-RDA, may be putative aberrantly methylated in leiomyoma tissue and their methylation statuses were examined in all five primary patients by MSP.

Methylation analysis of CpG islands in promoter region by MSP

It is considered that, hypermethylation rising in the core region of the 5' CGIs of genes closely correlated with transcriptional inactivation. Therefore, methylation statuses of the core regions in seven isolated genes, CHARC1 (8q24), FAM44B (5q35), FLJ33655 (1p34), HSUP (20q13), MLLT3 (9p12), SLC16A1 (1p12), and ZNF96 (6p22-21) (Table 1 and Fig. 1), were analyzed by MSP in five primary samples and immortalized human ovarian surface cell line (HOSE6-3) as a control. MSP was performed with a primer set specific to methylated or unmethylated sequence (M or U set, Table 2). The MSP results are shown in Fig. 2. The results did not reveal any aberrant DNA methylation of seven genes in 5 patients investigated.

DISCUSSION

In this study, we performed a genome-wide screen to identify aberrant DNA methylation in uterine leiomyoma by MS-RDA technology. A total of 192 clones were sequenced, and 7 genes with 5' CGIs, CHARC1, FAM44B, FLJ33655, HSUP, MLLT3, SLC16A1, and ZNF96 were isolated as putative aberrantly methyla-

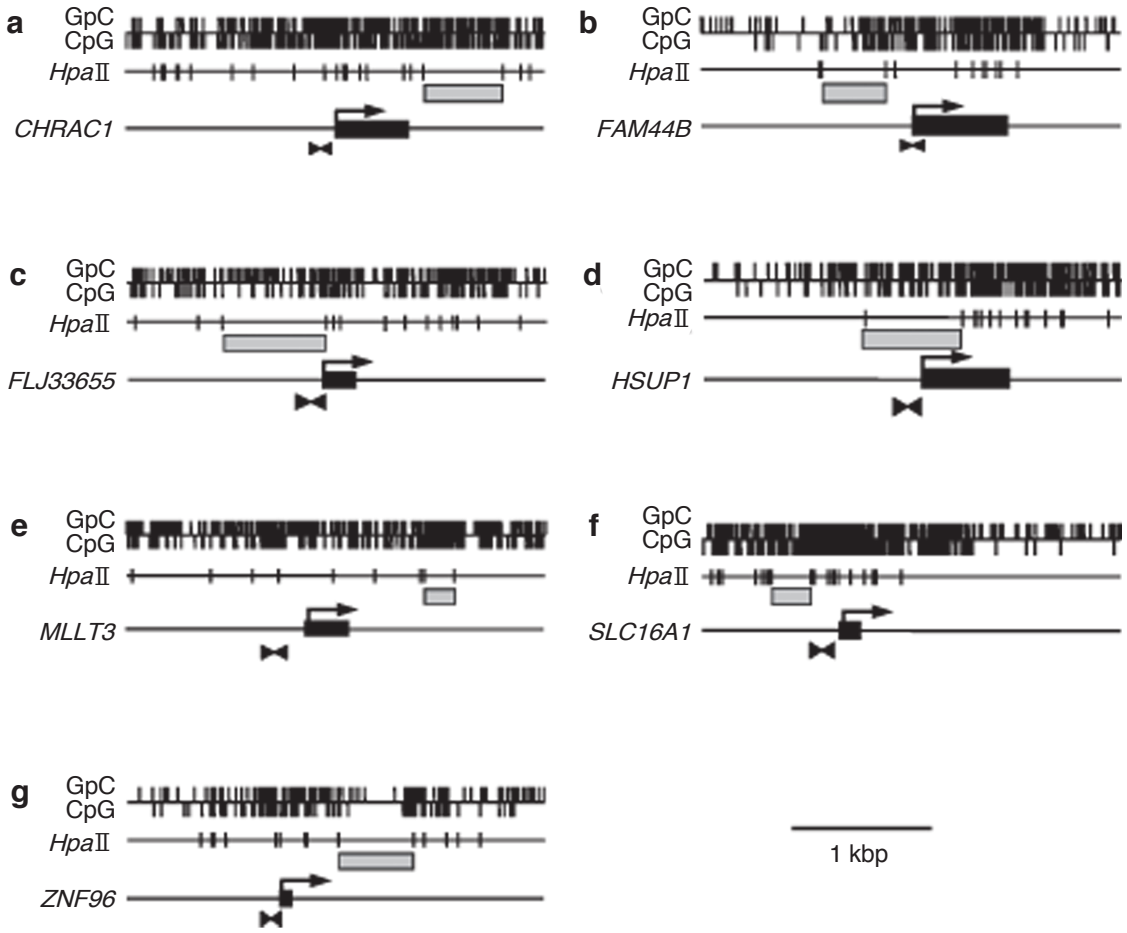

Fig. 1 Genomic structures around the seven analyzed CGIs in uterine leiomyomas. Vertical ticks show individual GpC sites (top), CpG sites (middle), and recognition sites (bottom) of restriction enzyme used for MS-RDA (HpaII). Gray boxes, DNA fragments isolated by MS-RDA; closed boxes, exons; arrowheads, MSP primers and arrows, transcription start sites and transcription directions.

tion in promoter regions (Table 1). These genes have not yet been reported that aberrantly methylated in any human tumors. MSP for 5 leiomyoma patients did not reveal aberrant promoter methylation in 7 genes (Fig. 2). These results permit two interpretations in this study. First, the isolated genes sporadically methylated in some CpG sites (CCGG), which recognized by HpaII, but not involved in whole CGIs. Second, may be that small samples examined in this study lead us with no findings. Though, our results in this study did not confirm DNA methylated in uterine leiomyoma, it did not exclude that epigenetic mechanisms may involve in the cause and development of uterine leiomyoma.

Previous investigators have shown epigenetic alteration of DNA methylation is expressed in uterine leiomyoma [20, 37]. Li *et al*. demonstrated that DNA global hypomethylation was detected in the uterine leiomyoma tissue using DNA methyl acceptance assay and immunohistochemistry staining with 5-methylcytidine antibody [20]. On the other hand, they also successfully screened two hypermethylated DNA fragments in uterine leiomyomas (NCBI access No. AZ081761 and No. AZ081762) [38]. As in cancer cells, global hypomethylation and local gene-specific hypermethylation can both be simultaneously expressed in uterine leiomyomas even though they are classified as a benign uterine disease.

Because over-expressions of estrogen-associated genes and various growth factors with mitogenic activity play a crucial role in prompting the growth of uterine leiomyomas [6], Li *et al*. postulated that the DNA global hypomethylation mechanism could contribute to elevating the expression of estrogen-associated genes and growth factors [20]. Gloudemans *et al*. also observed an inverse correlation between CpG methylation and expression of the insulin-like growth factor II (IGF-II) gene in malignant smooth muscle tissues [37]. In normal smooth muscle and in leiomyomas the IGF-II gene appeared to be methylated, while in leiomyosarcomas with IGF-II gene expression increasing, the overall methylation of IGF-II gene tended to be low or absent. MS-RDA can also identify the extensive hypomethylation of repetitive sequences, such as LINE1 [39]. In this study, we used myoma tissue DNA as driver, normal adjacent myometria DNA as tester, we cold not identify the hypomethylated DNA sequence in the leiomyoma. However, using the former DNA as tester, the latter DNA as driver should be possible to identify the hypomethylated DNA in the leiomyma.

On the other hand, increased DNMT1 and

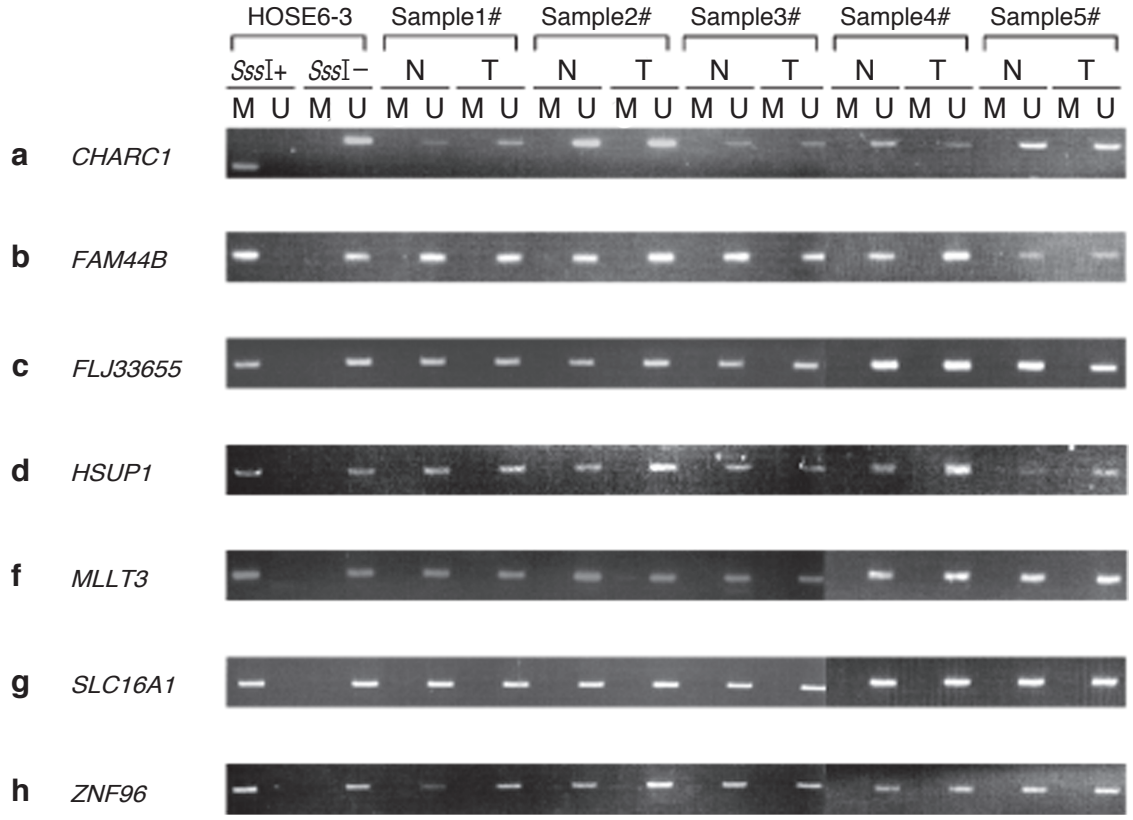

Fig. 2 Results of MSP for methylation analysis of 7 genes in 5 patients. HOSE 6-3: immortalized normal human ovarian surface epithelial cells; SssI+ : genomic DNA methylated by SssI methylase as fully methylated DNA control; SssI: genomic DNA not treated by SssI methylase as negative control. Samples 1-5: primary leiomyoma patients. N and T: normal adjacent myometria and tumor tissue, respectively. U and M: primer sets specific to unmethylated and methylated DNA molecules, respectively. No aberrant DNA methylation was found in 5 patients when analyzed for promoter methylation status of seven genes by MSP.

decreased DNMT3A and 3B expression were also revealed in uterine leiomyomas [20]. The DNMT family of enzymes catalyze the transfer of a methyl group to DNA. DNMT1 is responsible for maintenance and de novo DNA methylation, while DNMT3A and 3B are responsible for de novo DNA methylation, which refer to adding a new methyl group to unmethylated CpG sites [40]. It was reported that the expression of DNMT1, DNMT3A and 3B was elevated consistently in some cancer cells [41, 42]. While in aged cells, it showed a decrease of DNMT1 and an increase of DNMT3B [43]. Imbalanced expression of DNMT1, DNMT3A and 3B in human leiomyomas may indicate a different mechanism or proliferation efficiency in benign tumors, aged cells, and malignant tumors [20]. Taken together, a potential epigenetic mechanism plays its roles in the development of uterine leiomyomas.

In MS-RDA, use of cell lines, which can get a homogeneous population of cells, is highly recommended [25, 26]. In the present study, because few immortalized human leiomyoma and myometrial cell lines have been established, we used genomic DNA from an identical patient (case 1), whose leiomyoma tissue was used as the driver, and the adjacent myometria was used as the tester. Uterine leiomyomas are considered as monoclonal tumors that originate from smooth muscle cells [8]. Moreover, Abe *et al*. [32] also used genomic DNA of primary samples and cell lines in their MS-RDA analysis of neuroblastomas. Therefore, that use of genomic DNA of primary MS-RDA analysis is worth attempting.

Using MS-RDA can produce an abundance of DNA fragments that are unmethylated in the tester but putative and specifically methylated in the driver. It has been calculated that 104-105 CGIs can be screened by MS-RDA when compared with tumor and normal cells, and finally can distinguish 10-40 CGIs with different methylation status in a typical analysis [26]. We recently adopted MS-RDA technology to screen for CGIs aberrantly methylated in ovarian cancers, and isolated 33 CGIs that may be putative hypermethylation, and eventually successfully identified PRTFDC1 silencing and aberrant promoter methylation of GPR150, ITGA8, and HOXD11 in ovarian cancers [33]. In this study, we sequenced 192 clones and isolated 7 genes that putative aberrantly methylated in CGIs, but MSP did not find methyalted DNA in 5 primary uterine leiomyomas. Recently, Yamagata *et al*. demonstrated 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 using another genome-wide DNA methylation screening method, restriction landmark genomic scanning (RLGS) [44]. However, they only identified a new putative gene, GS20656, which showed an aberrant methylation status in uterine leiomyoma compared with myometrium [44]. It seemed possible that DNA methylation patterns differ among individuals. Therefore, to clarify the detailed difference in genome-wide DNA methylation status between uterine leiomyoma and normal myometrium, further analyses with larger samples are warranted using neither the MS-RDA nor the RLGS method.

In summary, this is an attempt of genome-wide screening to identify aberrant DNA methylation by MS-RDA in uterine leiomyoma. Although these 7 genes isolated by MS-RDA were not found to be aberrantly methylated in 5 primary samples, that does not rule out that epigenetic modifications of DNA methylation are involved in the cause and development of uterine leiomyomas. Larger populations of primary samples and more attempts using cell lines or primary monolayer cultures established from tissue samples are warranted to further elucidate these issues.

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