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—Original Article—

Potential Mechanisms of Aberrant DNA Hypomethylation on the X Chromosome in Uterine Leiomyomas

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Abstract. We recently found that aberrant DNA hypomethylation is more common on the X chromosome than on other chromosomes in uterine leiomyomas by genome-wide DNA methylation profiling. To investigate the mechanism of aberrant hypomethylation on the X chromosome in uterine leiomyomas, we analyzed methylome and transcriptome data from three cases of leiomyomas and the adjacent myometrium. We found that eleven of the aberrantly hypomethylated genes on the X chromosome were common to the three cases. None of these 11 genes were transcriptionally upregulated in the leiomyoma. However, one of them, *TSPYL2*, was hypomethylated in 68% of multiple leiomyoma specimens. The incidence of aberrant hypomethylation of *TSPYL2* was comparable to that of the *MED12* mutation (68%), which is known to be detected at a high frequency in uterine leiomyomas. We also analyzed the aberration of the X chromosome inactivation (XCI) mechanism in uterine leiomyomas. Hypomethylation was not enriched in the imprinted genes, suggesting that dysfunction of polycomb repressive complexes is not involved in the aberrant hypomethylation on the X chromosome. The expression analysis of XCI-related genes revealed that the *XIST* and *SATB1* expression was downregulated in 36% and 46% of 11 leiomyoma specimens, respectively, while the *HNRNPU* and *SMCHD1* expression was not altered. In conclusion, the aberration of XCI-related genes such as *SATB1* or *XIST* may be involved in aberrant hypomethylation on the X chromosome in a certain population of the patients with uterine leiomyomas. *TSPYL2* of the aberrantly hypomethylated genes on the X chromosome can be used as a biomarker of uterine leiomyomas.

Key words: DNA methylation, Uterine leiomyomas, X chromosome, X chromosome inactivation

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Uterine leiomyomas are the most common uterine tumors in reproductive-age women with a prevalence of 20–30% [1]. Although uterine leiomyomas are benign tumors, they cause severe pelvic pain, menorrhagia, dysmenorrhea, anemia, infertility and miscarriage [1, 2]. Therefore, the quality of life of women is significantly impaired. Little is known about the mechanism of the onset and development of uterine leiomyomas. While genetic factors such as African descent are associated with the risk of uterine leiomyomas, acquired factors such as a hormonal environment, nutrition and lifestyle also influence the risk of uterine leiomyomas; i.e., early menarche, high body mass index, meat diet and high blood pressure increase the risk, while the usage of oral contraceptive agents, giving birth, a vegetarian diet and smoking reduce the risk [3–6]. One possibility is that such factors cause epigenetic modifications in the genome that induce the onset and development of uterine leiomyomas.

Epigenetics is a mechanism controlling gene expression that does not involve a change in the primary DNA sequence. DNA methylation and histone modification play central roles in epigenetics. The DNA methylation pattern of each gene is different for each cell type [7, 8]. In addition, genome-wide epigenetic mutations (epimutations) caused by environmental factors such as long-term exposure to chemical compounds and abnormal nutritional balance have been implicated in several diseases, such as diabetes, autoimmune diseases and cancers [9–14].

We recently found that the aberrant DNA methylation occurs throughout the genome in uterine leiomyomas [15–17]. We identified 120 genes that are differently methylated and expressed between the leiomyoma and the adjacent normal myometrium, including a number of cancer-related genes [18]. Interestingly, we also found that aberrant hypomethylation was greater on the X chromosome than on other chromosomes in uterine leiomyomas [17, 18].

Genome- or chromosome-wide aberrant hypomethylation is thought to cause tumorigenesis through chromosomal instability (CIN) [10, 19]. The deletions and/or duplications of chromosomes accompanied by CIN have been implicated in the pathogenesis of malignant tumors [20]. For example, aberrant hypomethylation of the X chromosome is caused by loss of an inactive X chromosome followed by multiplication of an active X chromosomes in several female-related cancer cells such as breast, ovarian and cervical cancer cells [21–23]. By analyzing the polymorphism of microsatellites of the X chromosome, we demonstrated that uterine leiomyoma specimens harbored both parental X chromosomes [18]. In addition, the aberrant hypomethylation on the X chromosome seen in uterine leiomyomas is a phenomenon specific to uterine leiomyomas and has not been reported in other diseases so far. Because the aberrant hypomethylation on the X chromosome in uterine leiomyomas does

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not involve genetic factors such as duplication and deletion of both parental X chromosomes [18], the aberrant hypomethylation has been suggested to be caused by aberration of the X chromosome inactivation (XCI) mechanism.

XCI is initiated by RNA accumulation of *XIST*, a long noncoding RNA, throughout the inactive X chromosome, followed by histone modifications induced by polycomb repressive complexes (PRC) [24, 25]. We detected similar levels of *XIST* expression in the myometrium and leiomyoma [17]. Some other molecules have been implicated in XCI, including *SATB1* and *HNRNPU*, which function upstream of PRC in the XCI pathway, and *SMCHD1*, which functions downstream of PRC [25–29]. In this study, we focused on the feature of high frequency of aberrant hypomethylation on the X chromosome in uterine leiomyomas and identified the uterine leiomyoma-specific hypomethylated genes on the X chromosome. We also investigated whether XCI machineries are disturbed in uterine leiomyomas.

Uterine leiomyomas are benign tumor cells, and the morphological features of leiomyoma cells are poor; therefore, specific biomarkers to uterine leiomyomas have been required. Recently, mutations of the *Mediator complex subunit 12* (*MED12*) gene were detected in about 70% of uterine leiomyoma specimens, suggesting that the mutations of the *MED12* gene can be a uterine leiomyoma-specific biomarker [17, 30]. Since aberrant DNA hypomethylation is more common on the X chromosome than on other chromosomes in uterine leiomyomas, we also looked for hypomethylated genes on the X chromosome that can be used as uterine leiomyoma-specific biomarkers.

Materials and Methods

This study was approved by the Institutional Review Board of the Yamaguchi University Graduate School of Medicine. Informed consent was obtained from the patients before the collection of any samples. All experiments in which human tissues were handled were performed in accordance with tenets of the Declaration of Helsinki.

Sample preparations

Paired specimens of leiomyoma and adjacent normal myometrium were obtained from 22 Japanese women. Only women with a single leiomyoma nodule were selected to limit biological heterogeneity. The women underwent hysterectomy, and their ages were from 35 to 52 years old (mean ± SD; 44 ± 4.6). The diameter of leiomyomas ranged from 20 to 160 mm (mean ± SD; 70.7 ± 33.6 mm). None of the women had received previous treatment with sex steroid hormones or gonadotropin-releasing hormone analogs. Dissected specimens were immediately immersed in liquid nitrogen and stored at –80 C until DNA and RNA isolation.

DNA and RNA were isolated as described previously [31]. In brief, the genomic DNA was isolated by treatment with proteinase K (Qiagen, Hilden, Germany), followed by phenol/chloroform extraction and ethanol precipitation. Total RNA was isolated by treatment with ISOGEN reagent (Nippon Gene, Tokyo, Japan), followed by chloroform extraction and 2-propanol precipitation.

Sodium bisulfite genomic sequencing and combined bisulfite restriction analysis (COBRA)

Sodium bisulfite treatment was performed using an EpiTect Bisulfite kit (Qiagen) according to the conditions as follows: 95 C for 5 min, 65 C for 85 min, 95 C for 5 min and 65 C for 175 min. After sodium bisulfite treatment, PCR was performed using one unit of Biotaq HS DNA polymerase (Bioline, London, UK) and the primer sets shown in Table 4 under the thermocycling conditions (35 to 38 cycles of 95 C for 30 sec, 60 C for 30 sec and 72 C for 30 sec, with an initial step of 95 C for 10 min and a final step of 72 C for 7 min) as previously reported [18]. For bisulfite sequencing, the amplified PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). After sequencing reaction using a BigDye Terminator V3.1 Kit (Applied Biosystems, Foster city, CA, USA), sequencing was performed with a 3130xl Genetic Analyzer (Applied Biosystems). Bisulfite sequencing data was analyzed with QUMA (http://quma.cdb.riken.jp/) [32]. For COBRA, part of a PCR product was digested with *Taq*I (Takara, Tokyo, Japan) or *Hpy*CH4IV (New England Biolabs, Ipswich, MA, USA). Fragmentation of the treated and untreated PCR product was assessed by agarose gel electrophoresis.

RT-PCR

First strand cDNA was synthesized by random hexamers using a QuantiTect Reverse Transcription Kit (Qiagen) as previously reported [18]. The synthesized cDNA was amplified by PCR using one unit of Biotaq HS DNA polymerase and the primer sets listed in Table 4 under the thermocycling conditions (28 to 32 cycles of 95 C for 30 sec, 60 C for 30 sec and 72 C for 30 sec, with an initial step of 95 C for 10 min and a final step of 72 C for 7 min). *GAPDH* was used as an internal control for the quantity of the RNA used. The resulting products were subjected to agarose gel electrophoresis.

MED12 exon2 genomic sequencing

MED12 in leiomyoma and myometrium specimens was sequenced as described previously with some modifications [30, 33]. In brief, genomic PCR was performed using 1.25 units of PrimeSTAR GXL DNA Polymerase (Takara) and a primer set [30] shown in Table 4 under the thermocycling conditions (35 cycles of 98 C for 10 sec, 60 C for 15 sec, and 68 C for 45 sec). The amplified PCR products were purified using a QIAquick PCR Purification Kit (Qiagen), and sequenced with a 3130xl Genetic Analyzer. The obtained sequence chromatograms were analyzed manually.

Results

Identification of aberrantly hypomethylated genes on the X chromosome in uterine leiomyomas

We previously characterized the aberrant hypomethylation on the X chromosome using the methylome data from the illumina HumanMethylation450 BeadChip array (HumMeth450) and the transcriptome data on three cases of leiomyoma and adjacent myometrium [18] (these data are available at the Gene Expression Omnibus Web Site; http://www.ncbi.nlm.nih.gov/geo, under accession No. GSE45189). We defined genes as aberrantly methylated when they showed more than 30% hypermethylation or 30% hypomethylation in the leiomyoma compared with the myometrium [18]. Cases 1, 2 and 3 had 221, 134 and 178 aberrantly hypomethylated genes on the X chromosome, respectively, while in the whole genome,

Table 1. The number of aberrant hypomethylated genes on the X chromosome and in the whole genome in each leiomyoma specimen

	X chromosome			Whole genome	
	No. of hypomethylated genes (%)	No. of analyzed genes		No. of hypomethylated genes (%)	No. of analyzed genes
Case 1	221 (27.1)	814	Case 1	2386 (11.6)	20565
Case 2	134 (16.5)	814	Case 2	1327 (6.5)	20565
Case 3	178 (21.9)	814	Case 3	1487 (15.0)	20565

Table 2. Aberrantly hypomethylated genes on the X chromosome shared by the three cases

Gene	Location of the hypomethylated CpG
UPF3B	Ex1
OCRL	Upstream (< 200 bp)
IKBKG	Upstream (< 200 bp)
FOXR2	Ex1
TSPYL2	Int6
MID1IP1	Int1 (> 2.2 kb)
FRMD7	Ex1
MAP3K7IP3	Int1 (> 3 kb)
SRPX2	Ex1
PIN4	Ex4
GABRE	Int1 (< 300 bp)

Ex, exon; Int, intron.

2386, 1327 and 3078 genes were aberrantly hypomethylated in the leiomyoma in Cases 1, 2 and 3, respectively (Table 1). Because the numbers of genes analyzed in the HumMeth450 were 814 on the X chromosome and 20565 in the whole genome, the incidence of aberrant hypomethylation on the X chromosome was 16.5–27.1%, which was higher than that in the whole genome in uterine leiomyomas (6.5–15.0%) (Table 1).

Of the aberrantly hypomethylated genes on the X chromosome, eleven genes were common to the 3 cases (Table 2). Of the eleven genes, no genes were transcriptionally upregulated in the leiomyoma compared with the myometrium according to RT-PCR (data not shown) and previous transcriptome data [18].

Table 3. Human imprinted differentially methylated regions (DMRs) analyzed in this study

Locus	Methylated allele	Genomic location of DMR (UCSC hg19)	No. of CpGs on HumMeth450	References
DIRAS3	Maternal	chr1:68512494-68517474	31	34, 35
NAP1L5	Maternal	chr4:89618367-89620597	19	WAMIDEX, 36
PLAGL1	Maternal	chr6:144328817-144330002	19	34
IGF2R2	Maternal	chr6:160426265-160427502	4	35
GRB10 (primary)	Maternal	chr7:50849158-50851039	9	34
GRB10 (secondary)	Maternal	chr7:50861391-50861638	9	34
PEG10	Maternal	chr7:94284390-94286705	78	34
MEST	Maternal	chr7:130130115-130133159	62	WAMIDEX, 36
INPP5F_V2	Maternal	chr10:121577530-121578385	4	WAMIDEX, 36
H19/IGF2	Paternal	chr11:2019368-2023499	44	WAMIDEX, 36
KvDMR1	Maternal	chr11:2720129-2722236	32	34
WT1-AS	Maternal	chr11:32454216-32454721	2	WAMIDEX, 36
RB1	Maternal	chr13:48892536-48893957	5	35
DLK	Paternal	chr14:101192752-101193599	8	35
MEG3	Paternal	chr14:101292044-101292709	6	35
NDN	Maternal	chr15:23931560-23932547	5	WAMIDEX, 36
SNURF/SNRPN	Maternal	chr15:25199662-25201230	6	34
ZIM2	Maternal	chr19:57349677-57353037	35	34, 35
USP29	Unknown	chr19:57630340-57630725	10	35
MCTS2	Maternal	chr20:30135077-30135292	7	35
NNAT	Maternal	chr20:36147118-36151058	50	WAMIDEX, 36
L3MBTL	Maternal	chr20:42143024-42143698	8	WAMIDEX, 36
NESP	Paternal	chr20:57414509-57417327	15	34
NESP AS	Maternal	chr20:57426530-57427247	28	35
GNAS XL	Maternal	chr20:57428825-57431439	6	35
GNAS 1A	Maternal	chr20:57463453-57467939	62	35

Table 4. Information of the PCR primers in this study

#	Gene or DMR	Assay	Amplified region (UCSC hg19)	PCR primer	Product size (bp)	No. of CpGs	Restriction enzyme fragment size	References
1	*TSPYL2*	COBRA	chrX: 53,115,592-53,116,021	F: ATGGAGGAAAGAAAAAGTATTTTGAGTAA R: CCTCAATATAAATACATCCTCCAATTCA	430	5	TaqI 196 bp / 234 bp	
2	*OCRL*	COBRA	chrX: 128,673,840-128,674,262	F: GTAGAGGAGGAGTAAAGGTTTGGGT R: AAAAACTAAAAATCTAACCCAAAAAAAA	423	17	HpyCH4IV 295 bp / 128 bp	
3	*MED12*	Genomic PCR	chrX: 70,339,124-70,339,414	F: GCCCTTTCACCTTGTTCCTT R: TGTCCCTATAAGTCTTCCCAACC	291	–	–	30
4	KvDMR1	Bisulfite sequencing	chr11: 2,677,111-2,678,664	F: GTATGAGGTATTGGTTGGGTGTGAG R: CTAAAATCCCAAATCCTCAAAAATAAAC	454	40	–	
5	*GNAS XL* DMR	Bisulfite sequencing	chr20: 56,863,391-56,864,195	F: TTTTTTTAAGGTTAAGAAGGTATTTTTGG R: CCCTAAAACTTTCTAACAAATTCTTCCC	320	19	–	
6	IG-DMR	Bisulfite sequencing	chr14: 100,345,426-100,345,735	F: TTTTATTATTGAATTGGGTTTGTTAGT R: ACAATTCCTACTACAAAATTTCAACA	309	9	–	41
7	*HNRNPU*	RT-PCR	chr1: 245022081-245023721	F: TACAATGGAGAGTTTTGCTTTTCTT R: CAGTCTCACAGTTGCATGTTTTTAT	248	–	–	
8	*SATB1*	RT-PCR	chr3: 18458468-18462327	F: CTTGGGAGTACAGGTGCAAAAAT R: ATATCCTTTCTCACCAGCACAAAT	182	–	–	
9	*SMCHD1*	RT-PCR	chr18: 2726476+2729326	F: TGGCTTAAAAGAAGACTCACAGATT R: TCCAGAACTACTGCAATCTACAACA	241	–	–	
10	*XIST*	RT-PCR	chrX: 73,057,280-73,061,876	F: GTGGATAGAACACTGACTCTTGC R: GAGCCTAAGGAGACATGACTACT	719	–	–	
11	*GAPDH*	RT-PCR	chr12: 6,646,484-6,647,017	F: GTGGATAGAACACTGACTCTTGC R: GAGCCTAAGGAGACATGACTACT	341	–	–	

Methylation analysis of imprinted differentially methylated regions in the leiomyoma and myometrium

We investigated whether aberrant hypomethylation occurs in the genomic imprinted genes, in which the parental origin-specific expression is controlled in a manner similar to the XCI mechanism controlled by noncoding RNAs and PRC. Using the bisulfite sequencing method, we first examined the methylation status of three typical imprinted differentially methylated regions (DMRs) (KvDMR1, *GNAS XL* DMR and IG-DMR). The methylation levels of the three DMRs in the normal myometrium were 44.4–56.7%, nearly about 50% (Fig. 1A), suggesting that the parental allele-specific methylation of the DMRs is maintained. Because the methylation of the loci controlled under XCI and genomic imprinting is thought to occur monoallelically, aberrant methylation of the loci in the leiomyoma was defined as more than 15% hypomethylation or hypermethylation compared with the myometrium in this study. According to this definition, the uterine leiomyoma was aberrantly hypermethylated in the IG-DMR but not aberrantly hypomethylated in any other DMRs (Fig. 1A). Many human imprinted DMRs have been identified (WAMIDEX; https://atlas.genetics.kcl.ac.uk/) [34–36]. Table 3 shows 26 of these DMRs analyzed in this study. Fig. 1B compares the methylation rate of these DMRs between the leiomyoma and corresponding myometrium of 3 cases using the methylome data of the HumMeth450. Aberrant hypomethylation in the leiomyoma (white cells in Fig. 1B) was detected in two DMRs in Cases 1 and 2 and three DMRs in Case 3 (7.7–11.5% of the DMRs examined). This rate is comparable to the rate of aberrant hypomethylation in the whole genomes of the three cases (6.5–15.0%, Table 1), suggesting that aberrant hypomethylation is not more common in the imprinted DMRs in uterine leiomyomas.

Expression analysis of genes involved in the XCI mechanism

We examined whether there is any aberrant expression on the molecules regulating XCI (*XIST*, *HNRNPU, SATB1* and *SMCHD1*) in eleven cases. The mRNA expression of these genes was detected in the leiomyoma and corresponding myometrium in all cases (Fig. 2). Out of 11 cases, the relative expression levels of *XIST* mRNA were lower in 4 leiomyoma specimens (Cases 4, 5, 11 and 12) and higher in 1 specimen (Case 6) than those in the myometrium. The relative expression levels of *SATB1* mRNA was lower in 5 leiomyoma specimens (Cases 4, 5, 7, 11 and 12) and higher in 2 leiomyoma specimens (Cases 1 and 6). Thus, 5 out of 11 cases (45.5%) (Cases 4, 5, 6, 11 and 12) showed a common aberrant expression pattern in the *SATB1–XIST* system (Fig. 2). On the other hand, *HNRNPU* and *SMCHD1* showed no difference in mRNA levels between the leiomyoma and corresponding myometrium (Fig. 2).

Hot spots of the aberrantly hypomethylated genes on the X chromosome in uterine leiomyomas

To identify aberrantly hypomethylated genes that can be used as a biomarker of uterine leiomyomas, we searched the genes on the X chromosome that have hypomethylation specific to uterine leiomyomas. To this end, we focused on seven of the 11 genes in Table 2 (*UPF3B, OCRL, IKBKG, FOXR2, TSPYL2, MID1IP1* and *FRMD7*) that have a TCGA or ACGT site available for COBRA. The methylation rate was analyzed for multiple cases. Leiomyoma-specific

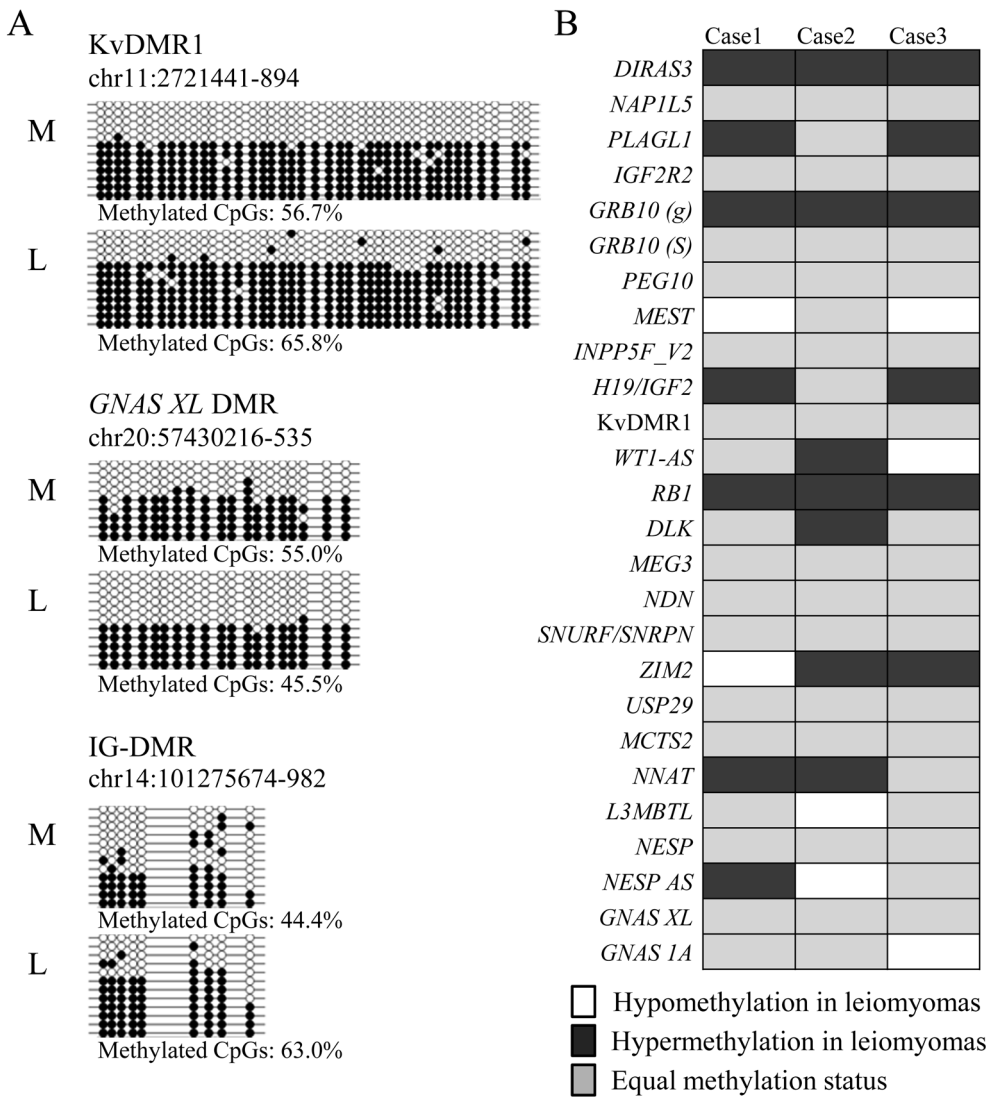

Fig. 1. Methylation status of imprinted differentially methylated regions (DMRs) in the leiomyoma and myometrium. A: Methylation analysis of the KvDMR1, *GNAS XL* DMR and IG-DMR by bisulfite sequencing in the leiomyoma and myometrium. The genomic position of each DMR indicates the region analyzed by bisulfite sequencing. Open and filled circles indicate the unmethylated and methylated CpG sites, respectively. Each horizontal line represents an individual clone. L and M in each case denote the leiomyoma and adjacent myometrium, respectively. Methylated CpGs represent the overall methylation percentage for each DMR (the number of methylated CpGs per number of total CpGs) in each specimen. B: The HumMeth450 data of three cases were searched for the methylation status of the 26 imprinted DMRs in the leiomyoma and myometrium. White and black boxes indicate hypomethylation or hypermethylation in the leiomyoma (more than 15% compared with the myometrium), respectively. The gray boxes indicate that the difference in the methylation rate between the leiomyoma and myometrium is less than 15%.

hypomethylation was observed in two genes, *Testis-specific Y-encoded-like protein 2 (TSPYL2)* and *Oculocerebrorenal syndrome of Lowe (OCRL)*. The methylation rate of the both genes in the myometrium was about 33% on average (*TSPYL2*, 24–40%; *OCRL*, 22–45%) and did not exceed 50% in any specimens (Fig. 3B), indicating that these loci are controlled under XCI. Aberrant hypomethylation in the leiomyoma was observed in 15 cases (68.2%, 15/22) in *TSPYL2* and in 11 cases (50%, 11/22) in *OCRL* (Fig. 3B).

MED12 mutation analysis

MED12 mutations were detected in 15 out of 22 (68.2%) leiomyoma specimens (Fig. 4A). *MED12* had a single nucleotide mutation in 14 and a deletion mutation in 1 of these 15 specimens. All of these single nucleotide mutations were previously reported to be hot spots in uterine leiomyomas, which were found in the 107th, 130th or 131st base of the coding region of *MED12* (Fig. 4B) [30]. These results indicate that the aberrant hypomethylation rate of the *TSPYL2* gene was comparable to that of the *MED12* mutation.

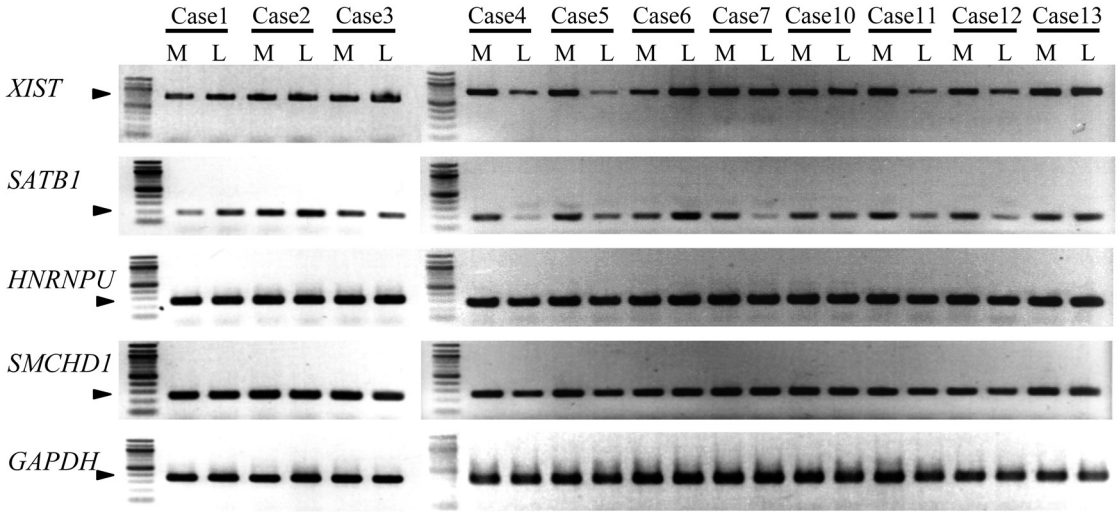

Fig. 2. mRNA expression of genes that are involved in the XCI mechanism. mRNA expressions of *XIST, SATB1, HNRNPU, SMCHD1* and *GAPDH* genes were measured by RT-PCR in 11 cases of the leiomyomas and adjacent myometrium. *GAPDH* was used as an internal control. Each arrowhead indicates the PCR product. L and M in each case indicate the leiomyoma and adjacent myometrium, respectively.

Discussion

Identification of aberrantly hypomethylated genes on the X chromosome in uterine leiomyomas

Aberrant hypomethylation was detected in many genes on the X chromosome in uterine leiomyomas, but changes in mRNA expression specific to uterine leiomyomas were not detected. These findings suggest that aberrant hypomethylation on the X chromosome in uterine leiomyomas randomly occurs in each individual. Therefore, it is unlikely that abnormal gene expression caused by aberrant hypomethylation on the X chromosome is involved in the pathogenesis of uterine leiomyomas. However, another possibility is that critical CpG sites, which are involved in gene expression related to the development of uterine leiomyomas, were not detected by the HumMeth450, which contains only a limited number of the CpG sites in the genome. Further studies are needed to clarify in more detail the methylation status on the X chromosome in uterine leiomyomas.

Aberration of the XCI mechanism in uterine leiomyomas

The incidence of aberrant hypomethylation in the imprint DMRs was comparable to that in the whole genome in uterine leiomyomas, suggesting that aberrant hypomethylation on the X chromosome is not caused by the dysfunction of PRC. We also compared the expression patterns of three genes that function upstream (*SATB1* and *HNRNPU*) and downstream (*SMCHD1*) of PRC in the XCI pathway. *HNRNPU* is involved in localization of *XIST* to the inactive X chromosome [29]. *SATB1* is involved in tethering the inactive X chromosome to the repressive core compartment for gene silencing [26]. *SMCHD1* has the potential role of maintaining the DNA methylation status of the inactive X chromosome [27, 28]. The expressions of *HNRNPU* and *SMCHD1* genes were not noticeably different between leiomyoma and normal myometrium. Alteration of the *XIST* and *SATB1* expression was observed in 5 and 7 out of 11 leiomyoma specimens, respectively, although it was not specific to the leiomyomas. Although the present study did not show clearly the dysfunction in the pathway of XCI in uterine leiomyomas, the aberrant expression of the *SATB1-XIST* system may be involved in aberrant hypomethylation on the X chromosome in a certain population of the patients with uterine leiomyomas.

Genetic and epigenetic biomarkers for uterine leiomyomas

To clarify the molecular mechanism of the pathogenesis of uterine leiomyomas, biomarkers are necessary to distinguish between leiomyoma cells and normal smooth muscle cells. So far, transcriptome analyses have not yet identified genes specifically expressed in uterine leiomyomas (for a review, see Arslan *et al.*, 2005 [37]). In addition, cytogenetic abnormalities in uterine leiomyomas, such as the trisomy of chromosome 12 [38], are too low in frequency to be used as biomarkers. Single nucleotide mutations in the vicinity of exon 2 of the *MED12* gene were detected in 159 out of 225 specimens (70%) obtained from 80 cases of uterine leiomyomas [30] and in 15 out of 22 cases (68.2%) in the present study. The *MED12* mutations appear in stem cells of uterine leiomyomas [33], indicating that they can serve as a diagnostic marker of uterine leiomyomas. As shown in the present study, other potential markers of uterine leiomyomas include aberrant hypomethylation of the *TSPYL2* and *OCRL* genes, with incidences in leiomyoma specimens of 68.2% and 50%, respectively. TSPYL2 is a nucleosome assembly protein and is required for cell cycle maintenance under stress conditions such as DNA damage [39]. OCRL is inositol 5-phosphatase, and loss of function of this protein results in oculocerebrorenal syndrome of Lowe [40]. Although the hyopomethylated region of the genes is unlikely to be involved in regulation of the gene expression, the incidence of aberrant hypomethylation, especially of *TSPYL2,* is comparable to

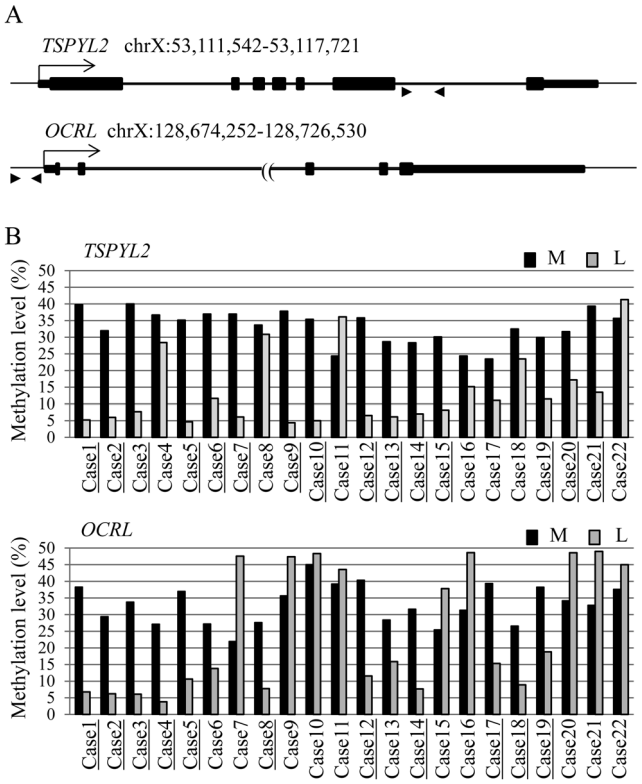

Fig. 3. Methylation analysis of *TSPYL2* and *OCRL* genes in the leiomyoma and adjacent myometrium in multiple cases. A: Schematic representation of the regions in the *TSPYL2* and *OCRL* genes analyzed for methylation levels. Positions on the genome show the genomic location of the genes. Arrows indicate the transcription start sites. Arrowheads indicate the position of the PCR primers used for COBRA. B: Methylation levels of *TSPYL2* and *OCRL* genes in 22 cases of the leiomyoma and adjacent myometrium. The bar shows the average of the percentage of methylation. The black and gray bars indicate myometrium (M) and leiomyoma (L), respectively. The underlined-cases indicate aberrant hypomethylation in the leiomyoma. Aberrant hypomethylation in the leiomyoma (lower than 15% compared with the myometrium) was observed in 15 cases (68.2%, 15/22) and 11 cases (50%, 11/22) in the *TSPYL2* and *OCRL* genes, respectively.

the incidence of the *MED12* mutations. Therefore, these epigenetic mutations should be useful markers of uterine leiomyoma cells in *in vitro* culture systems.

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A

Genotype	Number of cases (case #)	Nucleotide change
Point mutation	14 cases (#1, 2, 3, 4, 8, 10, 11, 12, 16, 17, 18, 19, 20, 22)	Detail in Fig. 4 B
Deletion	1 case (#5)	c.133_141del9
Normal	7 cases (#6, 7, 9, 13, 14, 15, 21)	–

B

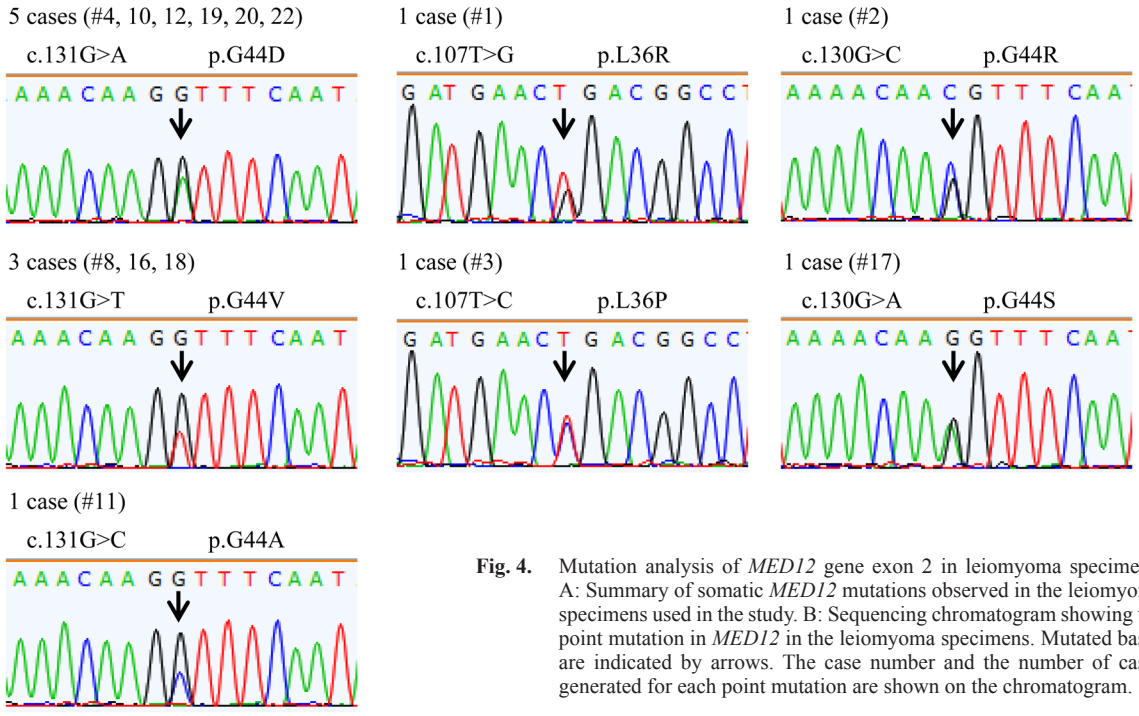

Fig. 4. Mutation analysis of *MED12* gene exon 2 in leiomyoma specimens. A: Summary of somatic *MED12* mutations observed in the leiomyoma specimens used in the study. B: Sequencing chromatogram showing the point mutation in *MED12* in the leiomyoma specimens. Mutated bases are indicated by arrows. The case number and the number of cases generated for each point mutation are shown on the chromatogram.

[Medline] [CrossRef]

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