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DNA hypomethylation and imbalanced expression of DNA methyltransferases (DNMT1, 3A, and 3B) in human uterine leiomyoma

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Abstract

Objective. Despite the high prevalence of uterine leiomyoma in women, little is known about the pathophysiology of this tumor. This study intends to define the epigenetic modulation of this tumor.

Methods. Twenty-three pairs of leiomyomas and their adjacent myometria were collected. Status of DNA global methylation was determined by using DNA methyl acceptance assay and immunohistochemistry staining with 5-methylcytidine antibody. MRNA level of DNA methyltransferases (DNMT1, 3A, and 3B) was assessed by quantitative real time PCR.

Results. DNA global hypomethylation was detected in the leiomyoma tissues as compared with the adjacent myometria. DNMT1 expression was increased in 47.5% and was equal in 47.5% in leiomyomas compared to the adjacent myometria. On the other hand, over 74% of cases showed decreased expression of DNMT3A and 3B in leiomyomas compared to the adjacent myometria.

Conclusion. Global hypomethylation and imbalanced expression of DNMTs in uterine leiomyoma suggested a potential mechanism of epigenetic modulation in the development of this tumor.

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Keywords: Uterine leiomyoma; Methylation; DNA methyltransferase; Real time PCR

Introduction

Uterine leiomyoma, commonly called fibroids or myoma, are benign tumors of myometrial origin. Uterine leiomyoma is a less-studied, but important, disease, which occurs in approximately 25% of all women and up to 70% of African American women [1–4]. Leiomyomas are well-differentiated, encapsulated monoclonal tumors with a distinct smooth-muscle phenotype. Enlarged tumors cause serious gynecological problems such as pelvic pain, menorrhagia, dysmenorrhea, reduced fertility, and recurrent pregnancy loss [2]. This disease is not only a leading cause of morbidity in women, but also a large cost factor in health care and a major indication for hysterectomies (>200,000 cases/year in the USA only) [5]. Leiomyomas are hormonally responsive tumors characterized by increased expression and function of estrogen receptor and estrogen-associated genes with few cytogenetic changes, such as translocation between chromosome 12 and 14, trisomy 12, rearrangement of short arm of chromosome 6 and of the long arm of chromosome 10, and deletions of chromosomes 3 and 7 [6,7]. The phenotype of the leiomyoma suggests that it resembles myometrium of pregnancy [6,7]. The question remains as to what are the underling causes for tumor development and hypersensitivity to hormones.

Epigenetic is defined as "heritable changes in gene expression that occur without a change in DNA sequence" [8,9]. DNA methylation is one of the important ways for

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epigenetic change to occur. DNA methylation occurs in CpG dinucleotides that are clustered frequently in regions of about 1–2 kb in length, called CpG islands, in or near the promoter and first exon regions of genes [10,11]. The mechanism of DNA methylation is not clear. Studies have focused on the role of different DNA methyltransferases, which transfer methyl groups from *S*-adenosylmethionines to C5 positions of cytosines. There are several different DNA methyltransferases, DNMT1, 2, 3A, 3B, and DNMT3L. DNMT1 is believed to be the "maintenance" enzyme, which maintains the methylation pattern during DNA replication [12,13], but is also believed to be involved in de novo DNA methylation [14]. DNMT2 conatins similar motifs of all other DNMTs, but dose not have methyltransferase activity in vitro [15]. The biological role of DNMT2 is unclear. DNMT3A/3B are known as de novo methyltransferases and have preferred target sites, which are different from DNMT1 [16,17]. DNMT3L does not have similar catalytic domain of other DNMTs nor any methyltransferase activity [18].

It is not clearly known how DNMTs silence gene expression. There are two widely accepted models: the first is that methylated DNA recruits methyl-CpG-binding protein (MBP) followed by recruiting of histone deacetylase (HDAC), which causes chromatin remodeling, and results in gene silencing [19]. The other model is that HDAC remodels chromatin first and allows DNMT1 to add methyl groups [20,21]. Both models indicated that DNA methyltransferases and histone deacetylase work together to silence gene expression. The noncatalytic domain of DNMT1 was further proven to be associated with the catalytic domain of HDAC1 directly [20]. In addition, the noncatalytic domain of DNMT3A is associated with HDAC1 and works as a transcriptional corepressor, which binds to RP58, a gene-specific transcriptional repressor. Thus, the association of RP58 and DNMT3A leads to silencing of specific genes which do not need the function of methyltransferase [22]. Alteration of methylation was considered an early event in carcinogenesis [10,11]. Previous work from our laboratory demonstrated that developmental estrogen exposure resulted in a permanant change in methylation status of an estrogen-responsive lactoferrin transferase gene [23].

Development of uterine leiomyoma is associated with estrogen exposure. Because of its benign, well-differentiated phenotype with few genetic changes, it is possible that the development of this tumor is modulated by epigenetic events. In this study, global DNA methylation and expression of different DNA methyltransferases, DNMT1, DNMT3A, and DNMT3B, were compared in uterine leiomyoma and adjacent myometrium from the same patient. Our results show global hypomethylation and differential expression of different DNMTs in leiomyoma tissues, suggesting a potential mechanism of epigenetic modulation in the development of this tumor.

Materials and methods

Tissue samples

Leiomyomas were obtained from 23 patients with leiomyoma undergoing hysterectomies at Tulane University Hospital and Clinics. Adjacent uterine myometria, 0.5–1 cm from the leiomyoma, were obtained from the same patient to serve as a control (designated as "normal"). There were 16 African American, 5 Caucasian, and 2 Hispanic women in this study. All the patients were premenopausal. Pathologically, of the 23 cases, 21 were usual leiomyoma and 2 were cellular leiomyoma. Leiomyoma samples with degenerative changes were excluded in this study.

DNA extraction and methyl acceptance assay

DNA was isolated from the uterine leiomyoma or myometrium using the QIAmp DNA Mini Kit (Qiagen, Valencia, CA) as per the manufacturer's protocol. Purified genomic DNA (1 μg) was used for measurement of DNA methylation status by methyl acceptance assay. DNA was incubated with 3 units of *Sss*I methylase (New England Biolabs Inc., Beverly, MA) and 1 μM ^{3}H-labeled *S*-adenosyl-L-methionine (79 Ci/mmol: SAM), 10 mM EDTA, 5 mM DTT, and 100 mM Tris–HCl (pH 8.2). The total volume of the mixture was 30 μl and it was incubated for 1 h at 37°C. The reaction was stopped by chilling on ice, and then 15 μl of reaction mixture was transferred onto DE 81 filter paper. The filter papers were washed by 0.5 M sodium phosphate buffer (pH 7.0) twice, followed by 70% ethanol and 100% ethanol and then air dried. The dried filter was transferred in 5 ml of scintillation fluid vial and radioactivity was measured by using a Beckman LS 9800 liquid scintillation system.

RNA extraction and reverse transcription

RNA was extracted using the Ultraspec RNA isolation kit (Biotecx Lab, TX). Reverse transcription reactions were carried out using the Gene Amp RNA PCR kit (Applied Biosystems, Branchburg, NJ). RNA (10 μg) from each sample was annealed with oligo dT primers at 70°C for 3 min and kept in 4°C. Extension reactions contained 5 mM $MgCl_2$, 1× PCR buffer II (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 125 μM dATP, dGTP, dCTP, and dTTP, 40 units of RNase inhibitors, 5 mM DTT, and 100 units of MuLV reverse transcriptase and annealing mixture in a final volume of 40 μl. The extension reaction was held in 42°C for 90 min and followed by 70°C for 10 min. The cDNA pool was stored at −70°C for the next PCR reactions.

Quantitative real time PCR

PCR was performed and analyzed using an iCycler iQ Real Time PCR detection system (Biorad, Hercules, CA).

Table 1
Primers, probes, and mean ΔC_T of DNMTs and GAPDH

Gene	Primers and probes (5′ to 3′)	Amplicon size (bp)	Amplification efficiency	r^2	Mean ΔC_T
GAPDH	F:CCCATGTTCGTCATGGGTGT R:TGGTCATGAGTCCTTCCACGATA P:CTGCACCACCAACTGCTTAG	145	0.92	0.99	N/A
DNMT1	F:GTTCTTCCTCCTGGAGAATGTCA R:GGGCCACGCCGTACTG P:TTGTCTCCTTCAAGCGCTCCATGGTC	138	0.87	0.96	9 ± 0.36
DNMT3A	F:CCTGTGGGAGCCTCAATGTTA R:TTCTTGCAGTTTTGGCACATTC P:CCTGGAACACCCCCTCTTCGTTGG	72	1.0	0.98	3 ± 0.62
DNMT3B	F:GACTCGAAGACGCACAGCTG R:CTCGGTCTTTGCCGTTGTTATAG P:AGCCACCTCTGACTACTGCCCCGC	97	1.0	0.98	3 ± 0.67

Note. F, forward primer; R, reverse primer; P, probe. r^2, correlation of three replicates of each data point in the efficiency calculation. Mean $\Delta C_T = C_{T\ \mathrm{DNMTs}} - C_{T\ \mathrm{GAPDH}}$. The mean ΔC_T's were derived from the average of 42 samples (20 from tumor and 22 from matched adjacent myometria) for each DNMT. Data are presented as means ± SE.

The sequences of primers and probes are listed in Table 1. All probes and primers were synthesized by IDT (Coralville, IA). Each intact fluorescent probe contained one reporter dye, 6-carboxyfluroescein (FAM), at the 5′ end and one quencher dye, Black Hole quencher (BHQ), at the 3′ end. During PCR cycles, Taq DNA polymerase, which also functioned as a 5′ exonuclease, cleaved quencher dye away from the probe that resulted in increased reporter fluorescence. The fluorescence intensity was detected with a CCD detector of iCycler (Biorad). The intensity was proportional to the amount of PCR products generated. Threshold cycle, C_T, was assigned to each sample when the fluorescent intensity exceeded 10 times the standard deviation of the baseline fluorescence threshold. Final quantification was done using the comparative C_T method and expressed as *n*-fold difference between tumor and normal.

PCR reactions were carried out in triplicate by adding 10 μl of 0.1× (for DNMT1) or 0.05× (for DNMT3A, 3B, and glyceraldehyde phosphate-3-dehydrogenase/GAPDH) diluted cDNA into 40 μl of PCR mixture. For amplification of DNMTs, the PCR mixture contained 1× PCR buffer II (50 mM KCl, 10 mM Tris–HCl, pH8.3), 5.5 mM $MgCl_2$, 0.6 mM dNTP mixture (Roche, CA), 800 to 1100 nM of each DNMT primer, 160 nM of each fluorescent probe, and 2.5 units of AmpliTaq Gold DNA polymerase. For amplification of the housekeeping gene, GAPDH, the PCR mixture contained 1× PCR buffer II (50 mM KCl, 10 mM Tris–HCl, pH8.3), 5 mM $MgCl_2$, 0.24 mM dNTP mixture (Roche, Foster City, CA), 350 nM of each primer, 80 nM fluorescent probe, and 2.5 units of AmpliTaq Gold DNA polymerase. PCR reactions were performed on a DNA thermal cycler, iCycler (Biorad). The reaction condition was 10 min at 95°C, 45 cycles of 15 s at 95°C, and 60 s at 60°C.

Single bands from PCR reactions of DNMT1, 3A, 3B, and GAPDH were cloned using a TA cloning kit from Invitrogen (Carlsbad, CA) and followed by sequence analysis. Selected clones with correct sequence of DNMTs and GAPDH were used as internal standards to verify plate-to-plate consistency. To avoid further differences between each run of PCR, tumor and normal tissues from the same patient were amplified on the same 96-well plate. Therefore, each set of PCR contains four different kinds of reaction mixture for four genes (DNMT1, 3A, 3B, and GAPDH) to amplify two pairs of cDNA from tumor and normal tissues of the same patient, as well as DNA clones of DNMT1, 3A, 3B, and GAPDH. All reactions were triplicate and randomly assigned on different wells of each plate.

The final threshold was adjusted in the exponential phase of amplification to have similar C_T of internal standards from different plates of reactions. The C_T of each sample, after being adjusted with the final threshold, was subtracted from the C_T of GAPDH to derive ΔC_T. The ΔC_T of tumor was compared to the ΔC_T of normal tissue and the difference was assigned as $\Delta\Delta C_T$. The relative level of expression of each DNMT between tumor and normal tissue was then calculated as $2^{-\Delta\Delta CT}$.

Four serial 10-fold dilutions of one tumor cDNA were amplified in triplicate to build up efficiency and standard curves of DNMTs and GAPDH. Standard curves were constructed by plotting measured C_T versus the logarithm of the initial amount of cDNA. Efficiency curves were built by plotting the increases of C_T after each 1:10 dilution vs the expected C_T.

Immunohistochemistry

Immunohistochemistry was used to assess the methylation status of the leiomyoma and the adjacent myometria. Paraffin-embedded, 4% formalin-fixed histological sections were made in 4 μm thickness. The sections were deparaffinized in xylene, and heated in 0.01 M citrate buffer for 15 min in a microwave oven. Then the slides were incubated in

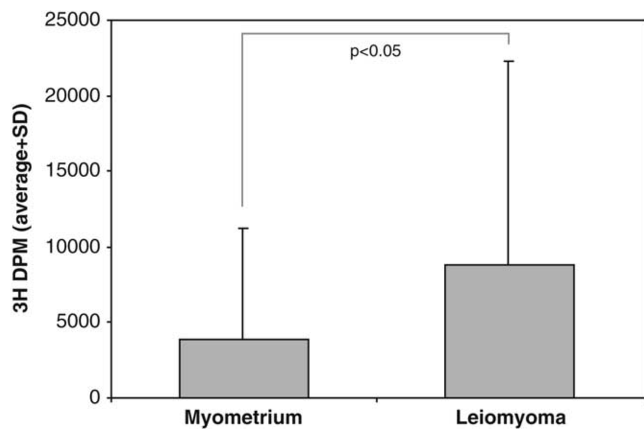

Fig. 1. Global DNA methylation measured by methyl-acceptance assay. Level of DNA methylation was determined using the in vitro methyl acceptance capacity assay and measured as DPM [*methyl*-^{3}H]SAM/1 μg DNA. The capacity of genomic DNA to accept radiolabeled methyl groups is inversely proportional to the level of DNA methylation.

methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase. Nonspecific binding was blocked with 10% normal goat serum for 30 min. Slides were incubated overnight at 4°C with monoclonal mouse antibodies against 5-methylcytidine (Eurogentec, Belgium, 4μg/ml) or control serum. After incubation with the primary antibodies, slides were washed and followed by applying secondary antibody (biotinylated rabbit anti-mouse; Vector Elite Kit) for 30 min at room temperature. After a quick rinse, the slides were incubated for an additional 30 min at room temperature with the ABC Elite complex (Vector Elite Kit). The slides were then incubated with chromagen-substrate reagent 3, 3′-diaminobenzidine (DAKO, Capinteria, CA) for 5 min at room temperature in the dark. All slides were counterstained in Harris' hematoxylin (Fisher, Pittsburgh, PA). Grading of immunohistochemistry slides was done using the quick score method [24]. In brief, this method evaluates both the average intensity of staining and the proportion of malignant cells staining. The average intensity was graded on a scale of 0 to 3 while the proportion of cells staining for each of the antigens was graded on a scale of 1 to 6. The two scores are multiplied to arrive at a quick score.

Results and discussion

Global hypomethylation of leiomyoma

DNA methyl acceptance assay reflects the capacity of genomic DNA to accept radiolabeled methyl groups in a methylase reaction mixture and is inversely proportional to the level of DNA methylation [25]. With this assay, our results showed that the incorporation ability of methyl donors was significantly higher in leiomyoma than that of the matched myometrium (Fig. 1). Thus, uterine leiomyomas are hypomethylated when compared to the normal myometria. Immunohistochemical staining with monoclonal 5-methylcytidine antibody was also used to assess the global methylation. This antibody binds with the modified 5-methylcytidine but not the nonmodified 5-cytidine. The staining was localized in nuclei of smooth muscle and vascular cells (Fig. 2A and B). The staining distribution was heterogeneous in both the leiomyoma and the myometrium; some cells showed strong staining, and some showed weak

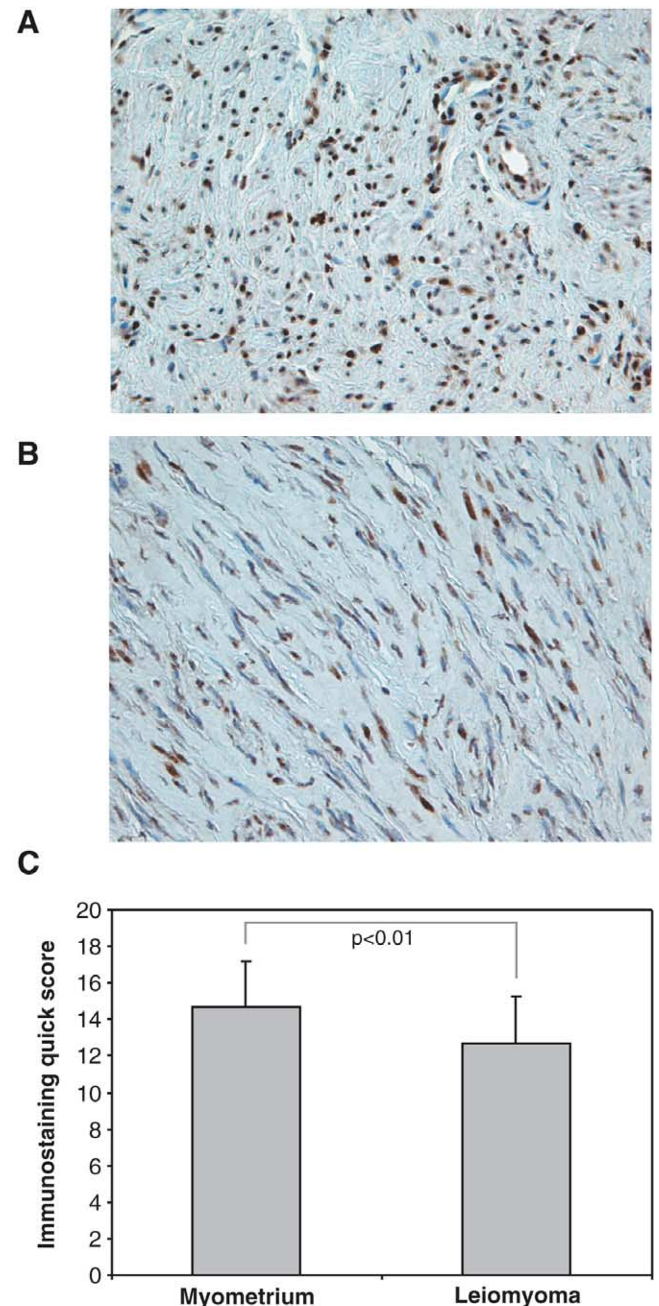

Fig. 2. Immunohistochemical staining of 5-methylcytidine in human uterine leiomyoma and adjacent myometrium. (A) Myometrium; (B) leiomyoma. (C) Quantitative staining intensity by quick score method. Quick score = intensity score × distribution score.

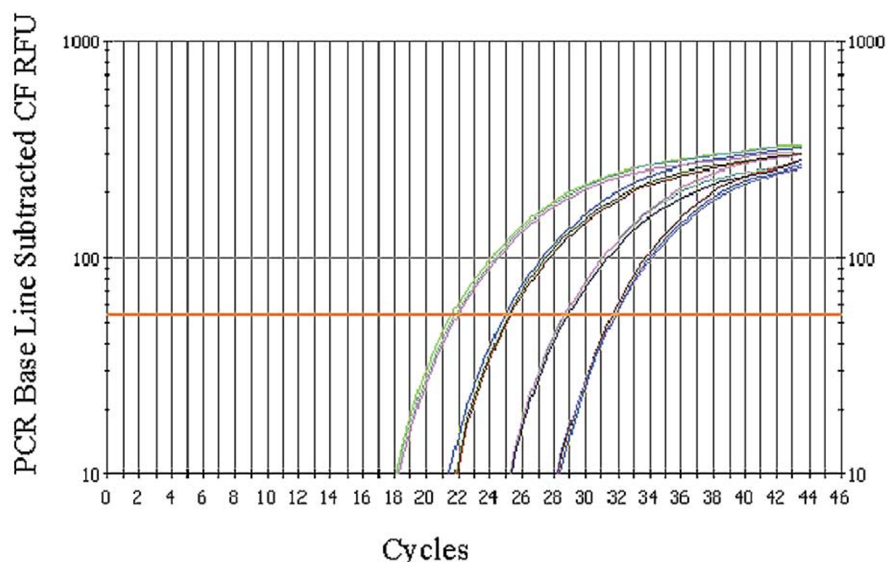

Fig. 3. Representative amplification plot of real time PCR after serial dilution from 1× to 200× (left to right). C_T is defined as the cycle threshold in which the fluorescent signal is higher than the selected threshold (indicated by the red line).

or undetectable staining. This reflected the diversity of methylation status in different cells. Using a quick score method, a semiquantitative intensity was achieved. The quick score was significantly lower in leiomyoma than that of the myometrium (Fig. 2C). This confirmed our result demonstrated by methyl acceptance assay that leiomyoma was hypomethylated as compared to the homogeneous myometrium.

It is very common that the level of the global methylation in tumor cells is lower than that in normal cells [26,27]. However, whether this hypomethylation is involved in tumor initiation or growth is unclear. While hypermethylation of a gene promoter usually leads to silencing gene expression, hypomethylation usually induces gene expression. Therefore, it is reasonable to postulate that global hypomethylation may contribute to the elevated estrogen-associated gene expression in the leiomyoma. Estrogen receptors and growth factors such as insulin-like growth factor (IGF) and epithelial growth factor (EGF) were found overexpressed in leiomyoma [6,28–30]. It is of great interest to know the association between hypomethylation and elevated expression of estrogen receptors and growth factors in the leiomyoma. On the other hand, hypermethylation of tumor suppressor genes such as p53, p16, and E-cadherin are frequently seen in tumor progression even if global hypomethylation existed in the tumors [10,11,31]. In fact, using a PCR-based method, methylation-sensitive restriction fingerprint (MSRF), we screened two hypermethylated fragments in human leiomyoma (NCBI access No. AZ081761 and No. AZ081762) [28]. Hence, imbalanced methylation status, i.e., coexistence of global hypomethylation and local gene-specific hypermethylation, may exist in leiomyoma.

Validation of real time PCR

The PCR amplification products of DNMT genes and internal control, GAPDH, revealed one single band at the expected molecular weight (data not shown). The PCR products were further sequence confirmed. The measured fluorescent signal showed typical PCR profiles: the signal remains low at early cycles followed by exponential increase and reached plateau phase at high cycle numbers (Fig. 3). The linear correlation between the C_T values and the initial amount of cDNA, diluted up to 1000-fold, confirmed the accuracy of the method in a wide detection range. The amplification efficiency of each gene is similar ($r^2 > 0.968$) (Table 1). Thus, the primers/probes were optimal for quantitative analysis of DNMTs in real time PCR.

Differential expression of DNMT1 relative to DNMT3A and 3B in uterine tissues

The expression level of DNMT1 is relatively lower than that of DNMT 3A and 3B in both leiomyoma and myometrium. The mean C_T values yield in DNMT1 amplifications was 9 ± 0.36, 6 C_T greater than the yields in DNMT3A and 3B amplifications (3 ± 0.62 and 3 ± 0.67 respectively, Table 1). Thus, the expression level of DNMT1 was lower than that of DNMT3A and 3B. This result is different from the known expression pattern of DNMTs in other tumor tissues, where higher or equal DNMT1 expression to DNMT3A and 3B has been observed [32]. The same set of primers and probes of DNMTs and GAPDH were tested using RNA from the Ishikawa cell line, derived from endometrial carcinoma. The expression level of DNMT1 achieved in this cell line was equal to DNMT3A and is 30 times higher than that of DNMT3B (data not shown). Thus,

Table 2
Relative expression of DNMT1, 3A, and 3B mRNA level in uterine leiomyoma compared to adjacent myometrium

	Tumor/control		DNMT1		DNMT3A		DNMT3B	
	Relative value	($2^{-\Delta\Delta CT}$)	No. of cases	%	No. of cases	%	No. of cases	%
Tumor < control	Less than 1/4	($<2^{-2}$)	1	5%	17	74%	18	79%
Tumor = control	1/2 to 2	(2^{-1} to 2^{1})	10	48%	5	22%	4	17%
Tumor > control	Greater than 4	($>2^{2}$)	10	47%	1	4%	1	4%
	Total		21	100%	23	100%	23	100%

Note. Relative values of leiomyoma (tumor) to myometrium (normal) of three DNMT genes were calculated based on the $2^{-\Delta\Delta CT}$.

the low expression level of DNMT1 in uterine tissue is not due to the low amplification efficiency of DNMT1. DNMT1 is believed to be responsible for copying methylation patterns following DNA synthesis. Low expression of DNMT1 is consistent with the observation of low proliferative activity in leiomyoma and myometrial cells [33]. Mitotic figures in both leiomyoma and the myometrium are very low. Most leiomyoma have less than 5 mitotic figures per 10 high-power fields, except for a rare type of leiomyoma, mitotically active leiomyoma [34].

Increased expression of DNMT1 and decreased expression of DNMT3A and 3B in leiomyoma

Despite the relative low expression of DNMT1 in both leiomyma and myometrium, using real time PCR, we were still able to distinguish the different expression levels of DNMT1 between leiomyomas and their adjacent normal myometria in most of the samples collected. Only two sets of samples with very low expression of DNMT1 were excluded from analysis because of inconclusive measurement in either normal or tumor samples. Thus, there were only 21 pairs of data for DNMT1 analysis. As shown in Table 2 and Fig. 4, 1 of 21 (5%) leiomyoma showed decreased expression of DNMT1, 10 of 21 (47.5%) showed increased expression of DNMT1, and the other 10 patients (47.5%) showed an equal amount of DNMT1 in the leiomyoma as compared to the matched myometria. Hence, most of the leiomyomas (95%) have equal or increased expression of DNMT1 as compared to the matched myometria. As mentioned above, DNMT1 is responsible for copying methylation patterns following DNA synthesis. The previous immunohistochemistry study showed that staining of Ki-67 was much higher in leiomyoma than that of the myometria throughout the menstrual cycle, suggesting that the leiomyoma has more growth potential [33,35]. An elevated DNMT1 level may reflect an elevated proliferative activity of leiomyoma cells. This conclusion is consistent with the study on human colon cancer where DNMT1 was reported to be proliferation dependent [36].

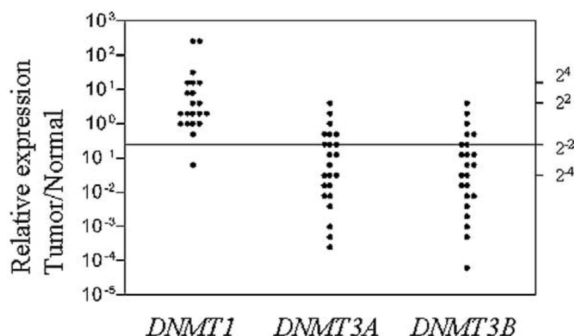

Fig. 4. Relative expression of DNMT1, 3A, and 3B mRNA level in uterine leiomyoma to adjacent myometrium. Relative value of leiomyoma (tumor) to myometrium (normal) of three DNMT genes were calculated based on the $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\ tumor} - \Delta C_{T\ normal} = (C_{T\ DNMT} - C_{T\ GAPDH})_{tumor} - (C_{T\ DNMT} - C_{T\ GAPDH})_{normal}$. There were 21 cases for DNMT1 and 23 for DNMT3A and 3B on this figure. Each dot represents one case. The solid lines at 2^2 and 2^{-2} represent the cutoff value. Only cases with absolute $\Delta\Delta C_T$ greater than or equal to 2 was considered to be different.

Increased expression of DNMT1 in leiomyoma is paradoxical to our finding of hypomethylation in leiomyoma. Similar findings from other studies also showed that some tumors overexpressed DNMT1 but had genomic hypomethylation [37,38]. In contrast, an in vitro study using overexpressed exogenous DNMT1 in mouse NIH3T3 cells found that global methylation was increased and followed by tumorigenic transformation [39]. One of the roles of DNMT1 is to maintain the methylation status in cells; thus the level of expression should reflect cell growth in normal or tumor cells. Overexpression of exogenous DNMT1 in cultured cells resulting in an increase of global methylation followed by tumorigenic transformation can be expected in an in vitro model. However, in an in vivo system, the increased DNMT1 may not be able to add extra methyl groups efficiently. With decreased expression of DNMT3A and 3B in leiomyoma, global DNA hypomethylation was reasonably expected in tumors where DNMT1 was increased.

As opposed to DNMT1, DNMT3A and 3B expression was decreased in leiomyoma as compared to their adjacent myometrium (Table 2 and Fig. 4). DNMT3A was decreased in 17 of 23 leiomyomas (74%), was equal in 5 of 23 leiomyomas (22%), and was increased in only 1 of 23 leiomyomas (4%); DNMT3B was decreased in 18 of 23 leiomyomas (79%), was equal in 4 of 23 leiomyomas (17%), and was increased in only 1 of 23 leiomyomas (4%). Decreased expression of DNMT3A and 3B are paralleled in

all except three cases. Activation of DNMT3A and 3B enzymes is considered to be responsible for de novo methylation, which refers to adding new methyl group to unmethylated sites. Decreased expression of both DNMT3A and 3B may contribute to the global hypomethylation of the tumor. In most cancer cells, the expression of DNMT1, 3A, and 3B was increased consistently [26,40–42]. However, there is no reported study of benign tumors, which may have different mechanisms or proliferation efficiency. Furthermore, the study showed decreased DNMT1 and increased DNMT3B on aged cells, which are low in replication efficiency [43]. Subnuclear distributions of DNMT1 are different from 3A and 3B. DNMT1 is associated with a nuclear replication site during S-phase, which fits the role of maintaining methylation, but DNMT3A and 3B are distributed throughout the nucleoplasm, not associated with S-phase replication [44]. Therefore, DNMT3A and 3B might use an alternative mechanism to methylate target DNA, and the level of DNMT1, 3A, and 3B could be induced or inhibited inconsistently in tumors. Imbalanced expression of DNMTs in uterine leiomyomas may be such a case.

In conclusion, global hypomethylation and differential expression of DNMT1, 3A, and 3B were revealed in uterine leiomyoma as compared with the matched normal myometria. A potential epigenetic mechanism of methylation modulation in the development of uterine leiomyoma is suggested. Further investigation of the expression DNMTs in patients with different hormonal status and age would help to illustrate the role of methylation in this tumor.

Acknowledgments

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