

***LATS2* promoter hypermethylation and its effect on gene expression in human breast cancer**

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Abstract. Tumor-specific promoter hypermethylation of large tumor suppressor, homolog 2 (*LATS2*), a tumor suppressor gene, has been investigated using methylation-specific polymerase chain reaction (MSP) assays in different types of human cancer producing conflicting results. The aim of the present study was to evaluate the methylation status of the *LATS2* promoter region using bisulfite sequencing with a next generation sequencer for breast cancer. In the 11 patients enrolled in the present study, the *LATS2* promoter methylation index (MI) was uniformly high in tumor and normal tissues of the breast (median, 84.0 and 87.4%, respectively). The presence of *LATS2* promoter hypermethylation was confirmed in isolated tumor cells and normal epithelial cells using the magnetic-activated cell sorting method. *In situ* hybridization for *LATS2* messenger RNA (mRNA) revealed that the mRNA expression of *LATS2* was higher in normal epithelial cells, compared with tumor cells, however, it was not significantly associated with *LATS2* MI. In 12 breast cancer cell (BCC) lines and two normal breast cell lines, the *LATS2* promoter was uniformly hypermethylated with no correlation between the mRNA expression of *LATS2* and the *LATS2* MI. In addition, treatment of the BCC lines with a demethylating reagent had minimal effect on the mRNA expression of *LATS2* in any of these cell lines. These results demonstrated that *LATS2* hypermethylation was not involved in silencing the mRNA expression of *LATS2* mRNA. The lower mRNA expression level of *LATS2* in tumor cells, compared with normal epithelial cells, suggested the possible involvement of downregulation in the mRNA expression of *LATS2* in the pathogenesis of breast cancer. Therefore, the conflicting results previously reported for *LATS2* promoter methylation in different types of cancer, detected using MSP assays may be attributable to the low fidelity of the MSP assay.

Introduction

Large tumor suppressor, homolog 2 (*LATS2*), is a tumor suppressor gene implicated in the regulation of cell proliferation, migration and apoptosis via several cell signaling pathways, particularly the Hippo, p53 and *LATS*-LIM domain kinase-actin pathways (1). Classically, a tumor suppressor gene is inactivated by a somatic mutation or promoter hypermethylation. Previous human cancer genome projects have revealed the presence of *LATS2* mutations in several types of human cancer, including breast cancer, however, its frequency is low at ~1% (2,3). Therefore, *LATS2* mutations are unlikely to be important in the pathogenesis of the majority of breast cancer cases. By contrast, hypermethylation of the *LATS2* promoter has been reported in a higher proportion of breast cancer (50%) (4), acute lymphoblastic leukemia (ALL) (24%) (5), astrocytoma (71.5%) (6), lung cancer (78.8%) (7), and nasopharyngeal cancer (36.7%) (8) cases, and the absence of *LATS2* hypermethylation has been confirmed in normal breast tissues (4) and normal brain tissues (6). In addition, the association between *LATS2* promoter hypermethylation and reduced messenger RNA (mRNA) expression levels of *LATS2* has been established in breast cancer (4), ALL (5) and astrocytoma (6). However, conflicting results have also been reported, namely, that *LATS2* promoter hypermethylation is rare in head and neck cancer (9), and that it is more common in normal tissues, compared with tumor tissues in nasopharyngeal cancer (8) and gastric cancer (10).

Investigations of the methylation of promoter regions are usually performed through bisulfite modification to convert the methylation status into a sequence difference. The present study carefully examined the *LATS2* promoter sequence following bisulfite modification and noted that several sequences with a high degree of homology to the *LATS2* promoter sequence were present in intron 2 (pseudo promoter sequence). As previous methylation studies of the *LATS2* promoter have used a methylation-specific polymerase chain reaction (MSP) assay, it is possible that the MSP assay may amplify the *LATS2* promoter sequence and also the pseudo promoter sequences in intron 2, resulting in the conflicting results of *LATS2* promoter methylation previously reported (4-10). This indicates that a more accurate technique is required for the investigation of *LATS2* promoter methylation. Advancements in sequence technology with the development of next generation sequencing

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(NGS), has made it possible to quantitatively determine, with high accuracy, the methylation status of a gene. It is anticipated that bisulfite sequencing with NGS can be used to evaluate the methylation status of the *LATS2* promoter more accurately than is possible using a conventional MSP assay.

The aim of the present study was, first, to evaluate the methylation status of the *LATS2* promoter region using bisulfite sequencing with NGS, and second, to clarify the correlation between *LATS2* promoter methylation and gene expression in breast cancer, using breast cancer cell (BCC) lines and breast cancer tissues.

Materials and methods

Patients and breast tumor samples. Pairs of tumor tissue and normal tissue samples were obtained from 11 patients with primary breast cancer, who underwent breast conserving surgery or mastectomy between 2001 and 2004 at Osaka University Hospital (Osaka, Japan). Patients treated with neoadjuvant chemotherapy and/or hormonal therapy were excluded. The clinicopathological characteristics of the patients recruited are summarized in Table I. The tissue samples were snap frozen in liquid nitrogen and stored at -80°C until use. The remaining surgical specimens were fixed with 10% buffered formaldehyde. The present study was approved by the Institutional Review Board for Clinical Research of Osaka University Hospital, and informed consent was obtained from each patient prior to surgery.

DNA extraction and sodium bisulfite treatment. Total DNA from cell lines was isolated using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the DNeasy[®] Blood and Tissue kit (Qiagen, Inc., Valencia, CA, USA) was used to extract total DNA from the breast tissues. The genomic DNA (1 µg) was treated with sodium bisulfite using an EpiTect[®] Bisulfite kit (Qiagen, Inc.).

Quantitative *LATS2* promoter methylation analysis using NGS. The GS Junior system (Roche Diagnostics, Basel, Switzerland) was used for the NGS methylation assay for region X (Fig. 1A) in accordance with the manufacturer's protocol, and data were analyzed using GS Amplicon Variant Analyzer software (version 2.7; Roche Diagnostics) (11). The *LATS2* gene sequence was obtained from the University of California Santa Cruz browser (NM_014572, December 2013; GRCh38/hg38; <https://genome.ucsc.edu>). The MI was calculated by dividing the number of reads of cytosine by the total number of reads at each CpG site. Sequence alignment between regions X and Z1-4 of the *LATS2* are shown in Fig. 1B. The NGS primers for region X, used for the frozen tissues and cell lines (NGS primer X-long; Fig. 1A), were as follows: Forward 5'-TTTTTTAGAAAGAAAATATATATGTTGGGGAGG-3' and reverse 5'-AAAAAAACCACCAAATTACCAACTAATAAC-3' (chr13: 21,061,921-21,062,405). For all 16 CpG sites, the average MI of 13 CpG sites was defined as the *LATS2* MI (2nd-14th CpG; Fig. 2A). The NGS primers for region X, used for the DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues (NGS primer X-short, Fig. 1A), were as follows: Forward 3'-TTATTGGGATAGTGAATTAATAATTAAG-5' and reverse 3'-AAAATTTCTCCAAATTAA

Table I. Clinicopathological characteristics of patients/tumors for comparison of the large tumor suppressor, homolog 2 methylation in paired breast tumor and normal breast tissues.

Characteristic	Patients, n (%)
Age (years)	
<50	4 (36)
≥50	7 (64)
Menopausal status	
Premenopausal	4 (36)
Postmenopausal	7 (64)
Tumor size (cm)	
≤2	3 (27)
>2	8 (73)
Lymph node metastasis	
Negative	8 (73)
Positive	3 (27)
Histological type	
Invasive ductal carcinoma	11 (100)
Other	0 (0)
Histological grade	
1 and 2	5 (45)
3	6 (55)
Estrogen receptor	
Negative	6 (45)
Positive	5 (55)
Progesterone receptor	
Negative	6 (45)
Positive	5 (55)
Human epidermal growth factor receptor 2	
Negative	7 (64)
Positive	4 (36)

TCAAATAATCT-5' (chr13: 21,061,987-21,062,136). The NGS short primer included six CpG sites corresponding to the 10-15th CpG, and the average MI of five CpG sites (10-14th CpG) was defined as the *LATS2* MI. The NGS primers for region Y (NGS primer Y; Fig. 1A) were as follows: Forward 5'-GGGTAAATATTTAAGTTTGGGGGTA-3' and reverse 5'-AACCCCTCACCTCCAAAAC-3' (chr13: 21061335-21061816), and the NGS primers for region Z1 (NGS primer Z1, Fig. 1A) were as follows: Forward 5'-TTATGTTTGGTTAGAAATTTTTTTT-3' and reverse 5'-ATCTACCAACACAATACCCAATACATA-3' (chr13: 21,039,732-21,040,116).

In situ hybridization (ISH) for *LATS2* mRNA. The QuantiGene[®]ViewRNA ISH Tissue Assay kit (Affymetrix, Inc., Santa Clara, CA, USA) was used in accordance with the manufacturer's protocol. FFPE sections (4 µm thick) of the tumor tissues were incubated for 10 min at 98°C with 200 ml 1X pretreatment solution (Affymetrix, Inc.), followed by protease digestion for 10 min. The *LATS2* View RNA[™] Probe set (Affymetrix, Inc.) was hybridized for 2 h at 40°C using

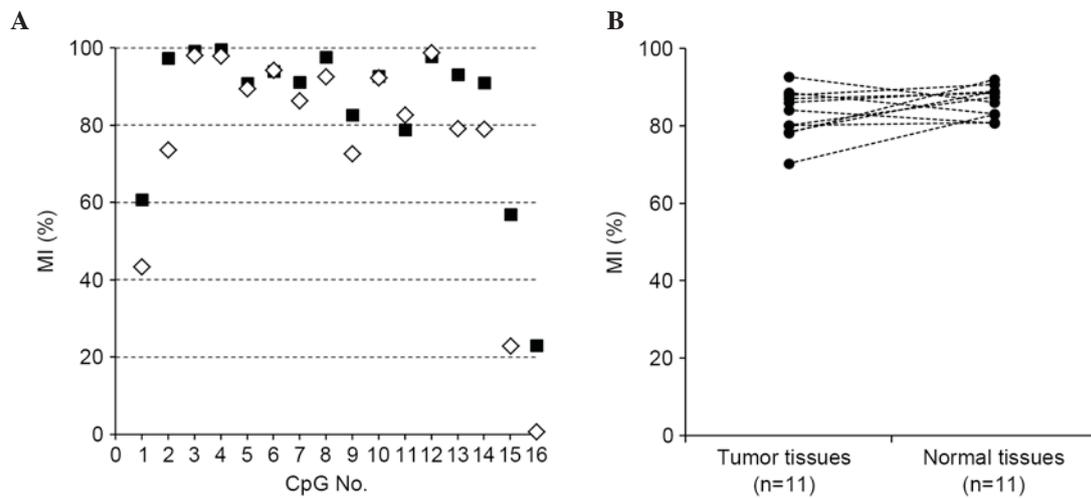


Figure 2. Methylation status of *LATS2* in breast cancer and normal breast tissues. (A) Representative results for *LATS2* MI in paired normal breast tissues (white diamonds) and cancer tissues (black squares). The average MI percentages of CpG sites 2-14 were used for statistical analyses. (B) Comparison of *LATS2* MI in paired normal breast and cancer tissues. *LATS2*, large tumor suppressor, homolog 2; MI, methylation index.

a Hybridizer (Dako, Glostrup, Denmark). ISH images were captured using a fluorescent microscope (BX63; Olympus, Tokyo, Japan). Signal intensity was semi-quantitatively classified into four categories (0, negative; 1+, weak; 2+, intermediate; and 3+, strong) based on the number of cytoplasmic fluorescent dots in five non-overlapping fields at high-power magnification (x400).

Isolation of breast tumor cells using magnetic-activated cell sorting (MACS). The breast cancer cells and normal epithelial cells were isolated from the FFPE tissues using the MACS method with the EasySep Human EpCAM Positive Selection Cocktail, the EasySep Human MUC1 Positive Selection Cocktail and EasySep Magnetic Particles (Stemcell Technologies, Inc., Vancouver, BC, Canada), as previously described (12). Total DNA was extracted from these isolated cells using the QIAamp[®] DNA FFPE Tissue kit (Qiagen, Inc.).

Cell lines and analysis of demethylation using 5-aza-2'-deoxycytidine (5-aza). The 12 BCC lines (MCF7, ZR75-1, T47D, ZR75-30, MDA-MB-361, BT474, SKBR3, AU565, MDA-MB-453, MDA-MB-231, MDA-MB-468 and BT-20) and two normal breast epithelial cell lines (MCF10A and HMEC) were cultured according to the culture guides of the American Type Culture Collection (Manassas, VA, USA). For the investigation of demethylation, the cultured cells were seeded into a six-well plate at a density of 1.5×10^5 cells/well, and treated with $5 \mu\text{mol/l}$ 5-aza (Sigma-Aldrich; Merck Millipore) or with dimethylsulfoxide as a control for 72 h at 37°C , with the medium replaced every 24 h.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from the cell lines using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and $1 \mu\text{g}$ of total RNA was reverse-transcribed into single strand cDNA using random primers and the ReverTra Ace[®] qPCR RT kit (Toyobo Co., Ltd., Osaka, Japan). *LATS2* and *GAPDH* TaqMan[®] Gene Expression Assays (Hs00324396_m1 and Hs02758991_g1; catalog nos.,

4351372 and 4331182, respectively; Applied Biosystems; Thermo Fisher Scientific, Inc.) were used for qPCR on the LightCycler 480 Real-time PCR System (Roche Diagnostics). The qPCR primers and probes used for the measurement of *LATS2* and *GAPDH* mRNA expression were designed by the Applied Biosystems (Thermo Fisher Scientific, Inc.). The cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 60°C for 60 sec and 50°C for 10 sec. Absolute mRNA expression levels of *LATS2* and *GAPDH* were determined by the standard amplification curves obtained by serially diluted PCR products with known concentrations. The expression of *LATS2* was normalized to that of *GAPDH*, and each assay was performed in duplicate. The result for each of the 5-aza treated BCC lines was normalized with that of its untreated control.

Statistical analysis. The JMP statistical software package (version 10; SAS Institute, Cary, NC, USA) was used for statistical analyses. The association between the expression of *LATS2* and MI was assessed using the Kruskal-Wallis test. The Wilcoxon signed-rank test was used for comparison of the expression levels of *LATS2* in the cancer and normal tissues. All statistical analyses were two-sided and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

*Methylation status of the *LATS2* promoter in breast tumor and normal tissues.* In the present study, 11 pairs of frozen breast tumor and normal tissues were analyzed using an NGS-based methylation assay which involved bisulfite sequencing with NGS. Representative results for the *LATS2* MI at each CpG site of region X are shown in Fig. 2A, demonstrating that its methylation was uniformly high in the tumor and normal tissues (median 84.0 and 87.4%, respectively; Fig. 2B). The tumor cells and normal epithelial cells were then isolated using the MACS method from five pairs of FFPE tumor and normal tissues, respectively, and subjected to the NGS-based methylation assay. The isolated tumor cells exhibited high MI

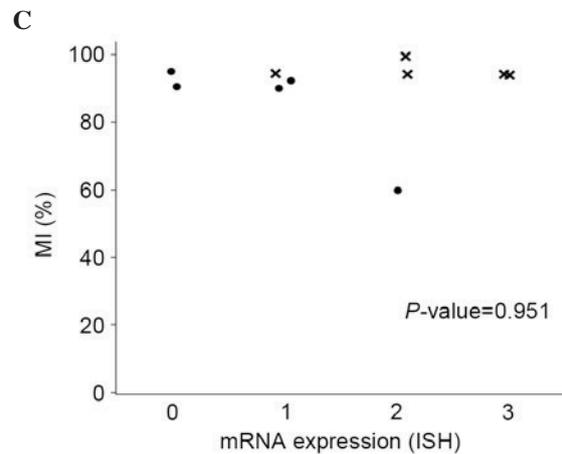
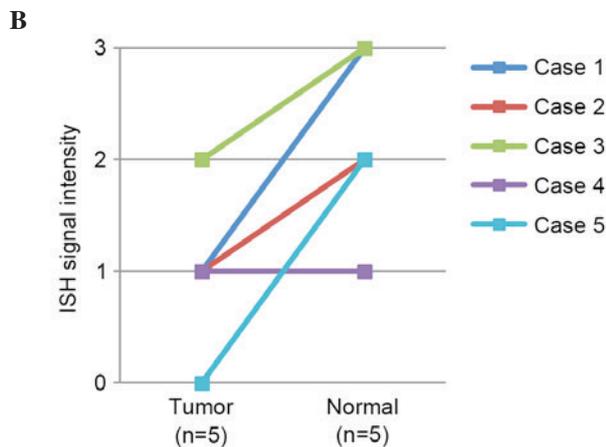
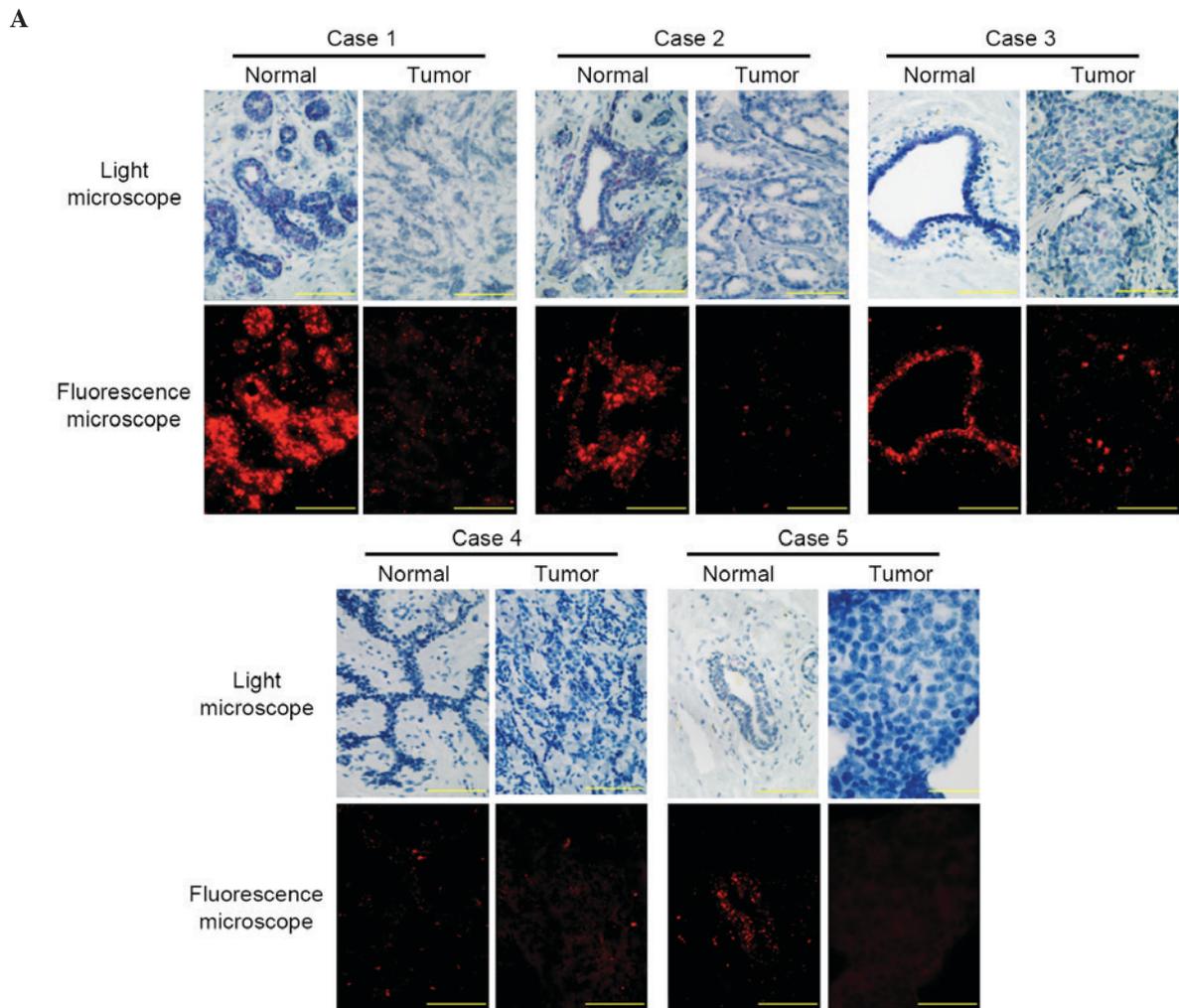


Figure 3. Representative results of ISH analysis of the mRNA expression of *LATS2* in breast cancer tissues. (A) Results of ISH analysis of the mRNA expression of *LATS2* in five paired breast cancer and normal tissues. Microscopic images were captured under a light microscope and a fluorescent microscope (magnification, x400; scale bars=100 μ m). (B) Comparison of ISH signal intensity in paired normal breast and cancer tissues. (C) Correlation between the mRNA expression of *LATS2* and MI in normal breast tissue (x) and cancer tissues (black circles). ISH, *in situ* hybridization; *LATS2*, large tumor suppressor, homolog 2; MI, methylation index.

(median, 90.6%; range, 59.9-95.1%), as did the normal epithelial cells (median, 94.4%; range, 94.1-99.7%).

mRNA expression of LATS2 and its correlation with LATS2 promoter methylation in breast tumor and normal tissues.

ISH for *LATS2* mRNA was used to examine the five pairs of FFPE breast tumor and normal tissues (Fig. 3A). In four of the five pairs, the mRNA expression of *LATS2* was higher in the normal breast epithelial cells, compared with the tumor cells (Fig. 3B). No significant correlation was observed between

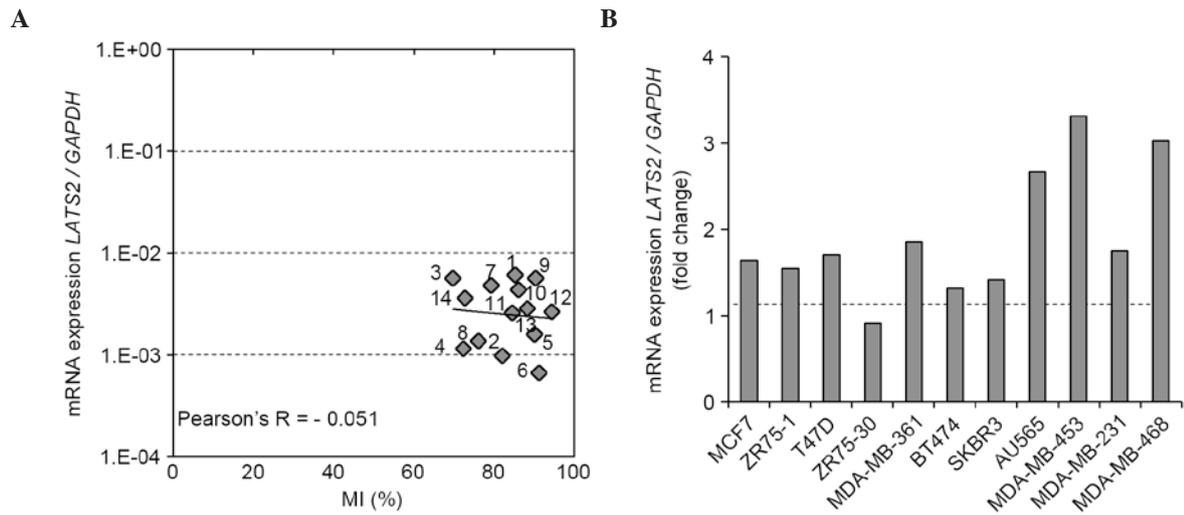


Figure 4. *LATS2* methylation status of region X, the mRNA expression of *LATS2* in breast cancer cell lines and the effect of demethylating reagent on mRNA expression. (A) Correlation between *LATS2* MI and mRNA expression. The MI of *LATS2* (region X) was determined using a next generation sequencing-based methylation assay and was compared with the mRNA expression of *LATS2* in 12 BCC lines and two normal breast epithelial cell lines (MCF10A and HMEC). (B) Effect of 5-aza treatment on the mRNA expression of *LATS2*. The mRNA expression of *LATS2* was evaluated following treatment with 5-aza or vehicle (dimethyl sulfoxide) in 11 BCC lines. The fold changes in the mRNA expression of *LATS2* following 5-aza treatment are shown. *LATS2*, large tumor suppressor, homolog 2; BCC, breast cancer cell; MI, methylation index; 5-aza, 5-aza-2'-deoxycytidine; 1, MCF7; 2, ZR75-1; 3, T47D; 4, ZR75-30; 5, MDA-MB-361; 6, BT474; 7, SKBR3; 8, AU565; 9, MDA-MB-453; 10, MDA-MB-231; 11, MDA-MB-468; 12, BT-20; 13, MCF10A; 14, HMEC.

the mRNA expression of *LATS2* and the *LATS2* MI ($P=0.951$; Fig. 3C).

Promoter methylation of *LATS2* and its effect on gene expression in BCC lines. To examine the methylation status of *LATS2* region X in BCC lines, NGS-based methylation assays were performed using 12 BCC lines and two normal breast cell lines. Similar to the findings for the breast tumor and normal tissues, the *LATS2* promoter was found to be uniformly hypermethylated in all BCC lines and HMEC cells (Fig. 4A). Subsequently, the correlation between *LATS2* promoter methylation and the mRNA expression of *LATS2* was investigated using RT-qPCR analysis. No inverse correlation was observed between the mRNA expression of *LATS2* and *LATS2* MI (Pearson's correlation coefficient -0.051 ; Fig. 4A). Treatment of these BCC lines with a demethylating reagent ($5 \mu\text{M}$ 5-aza) had minimal effect on the mRNA expression of *LATS2* in any of the cell lines, suggesting that the mRNA expression of *LATS2* was not directly regulated by methylation of its promoter (Fig. 4B). NGS-based methylation assays using eight BCC lines and two normal breast cell lines were then performed for region Y in the CG-rich region located 333 bp downstream of region X, and for region Z1, which was most homologous to region X in intron 2 (Fig. 1A). Region Y was completely unmethylated and region Z1 showed a high level of methylation in all the cell lines. Neither the methylation status of regions Y or Z1 was correlated with the mRNA expression of *LATS2* (Fig. 5A and B).

Discussion

The present study investigated *LATS2* promoter methylation (region X; Fig. 1A) using NGS-based methylation assays, a more accurate method, compared with MSP assays. It was found that the *LATS2* promoter exhibited a high level of

methylation in breast tumor cells and normal breast epithelial cells, and that the mRNA expression of *LATS2*, determined using ISH, did not correlate with *LATS2* promoter methylation. In addition, the *LATS2* promoter exhibited high levels of methylation in all 12 BCC lines, regardless of their subtypes, and in two normal breast cell lines. Furthermore, it was established that treatment of BCC lines with a demethylating reagent had minimal effect on reactivation of the mRNA expression of *LATS2*. Taken together, these results demonstrated that *LATS2* promoter hypermethylation was not implicated in the silencing of *LATS2* in breast cancer and is unlikely to be important in the pathogenesis of breast cancer.

The present study showed that the mRNA expression of *LATS2*, determined using ISH, was lower in tumor cells, compared with normal breast epithelial cells, suggesting the possible involvement of the downregulation of *LATS2* mRNA in the pathogenesis of breast cancer. Such a reduction in the mRNA expression of *LATS2* is likely to be induced by mechanisms other than promoter hypermethylation. Previously, several microRNAs, including microRNA (miR)93 in breast cancer (13), miR25 in ovarian cancer (14) and miR31 in lung (15) and endometrial cancer (16), have been reported to directly target the *LATS2* gene and reduce its anti-oncogenic effect. A previous study identified a *LATS2* mutation, which can reduce the expression of the gene in lung cancer, however, such a mutation in breast cancer remains to be elucidated (2,3). Furthermore, the mechanism underlying the downregulated mRNA expression of *LATS2* in tumor cells requires more detailed investigation.

Until now, reports regarding *LATS2* promoter hypermethylation have been inconsistent in their support for tumor-specific *LATS2* hypermethylation in various types of human cancer (4-9; Table II). Such inconsistency is most likely attributable to the low fidelity of the MSP assay used in these previous studies. A schematic representation of the *LATS2* genome is shown in

Table II. Summary of previous studies examining *LATS2* methylation in human cancer.

Author (year)	Type of Cancer	Tissue	n	<i>LATS2</i> methylation positive rate (%) ^a	Refs.
Visser and Yang (2010)	Breast	T	30	50	(1)
		N	6	0	
Steinmann <i>et al</i> (2009)	Head and neck	T	54	80	(9)
		N	23	90	
Zhang <i>et al</i> (2010)	Nasopharyngeal	T	30	37	(8)
		N	23	100	
Jiang <i>et al</i> (2006)	Astrocytoma	T	88	72	(6)
		N	10	0	
Sasaki <i>et al</i> (2010)	Lung	T	203	79	(7)
		N	-	-	
Jiménez-Velasco <i>et al</i> (2005)	ALL	T	66	24	(5)
		N	-	-	

^aDetermined using methylation-specific polymerase chain reaction assays. *LATS2*, large tumor suppressor, homolog 2; T, tumor; N, normal; ALL, acute lymphoblastic leukemia.

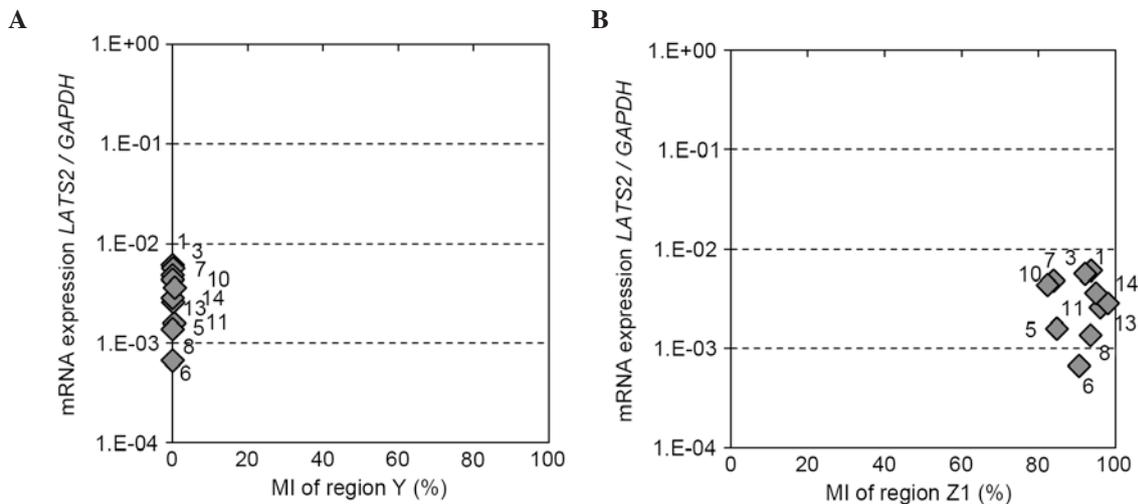


Figure 5. *LATS2* methylation status of regions Y and Z1, and mRNA expression of *LATS2* in breast cancer cell lines. Correlation between the *LATS2* MI of (A) region Y or (B) region Z1 and mRNA expression. *LATS2* MI was determined using a next generation sequencing-based methylation assay and was compared with the mRNA expression of *LATS2* in eight breast cancer cell lines and two normal breast epithelial cell lines (MCF10A and HMEC). *LATS2*, large tumor suppressor, homolog 2; MI, methylation index; 1, MCF7; 3, T47D; 5, MDA-MB-361; 6, BT474; 7, SKBR3; 8, AU565; 10, MDA-MB-231; 11, MDA-MB-468; 13, MCF10A; 14, HMEC.

Fig. 1A. A careful survey of the *LATS2* promoter sequence following bisulfite modification revealed the presence of several sequences (region Z1-Z4 in Fig. 1A) in intron 2, which exhibit a level of homology with the *LATS2* promoter sequence. The most homologous sequence was region Z1, which was 92% identical to region X (Fig. 1A), and located 22,024 bp downstream of region X. The alignment of the region X and Z1-Z4 sequences following bisulfite modification is shown in Fig. 1B. The MSP primers used in our previous study (4) were designed on the basis of Sanger bisulfite sequencing. The primers used for bidirectional Sanger sequencing hybridize to regions X and Z, and the sizes of the amplicons from the two regions were almost identical at 212 bp in region X and 211 or 212 bp in region Z.

Therefore, Sanger sequencing may produce varied sequence results for regions X and Z, leading to the faulty design of MSP primers (Table III). With such MSP primers, it is possible that the two regions are amplified at varying ratios depending on the PCR conditions, which raising concerns as to whether the assessment of *LATS2* promoter methylation is accurate. By contrast, with NGS, accurate sequencing can be performed with a single base resolution, which avoids the possible misreading of similar sequences. In addition, the NGS primers were designed outside region X to avoid illegitimate hybridization (Fig. 1A). Therefore, the NGS-based methylation assay was considered sufficiently accurate to provide a true assessment of the methylation status of the *LATS2* promoter.

Table III. Primer sequences used in previous studies.

Author (year)	Primer	Concordance rate (%)	Refs.
Sanger sequencing primers			
Takahashi <i>et al.</i> (2005)	F 5'-TTTTGAGATGGAGTTTTGT-3' R 5'-AATTCAAACCAACCTAACC-3'	- -	(4)
Methylation-specific primers			
Takahashi <i>et al.</i> (2005);			(4)
Jiang <i>et al.</i> , (2006);			(6)
Sasaki <i>et al.</i> , (2010);			(7)
Zhang <i>et al.</i> , (2010)			(8)
	M-F 5'-ATTTCGGTTTATTGTAATTTTC-3' Region -X: 21,062,261-21,062,240 Region -Z1: 21,040,026-21,040,005 Region -Z2: 21,029,744-21,029,723 Region -Z3: 21,023,926-21,023,905 Region -Z4: 21,019,927-21,019,906	82 95 91 82 73	
	M-R 5'-AACCAACATAATAAAACCCCG-3' Region -X: 21,062,138-21,062,117 Region -Z1: 21,039,903-21,039,883 Region -Z2: 21,029,620-21,029,599 Region -Z3: 21,023,804-21,023,783 Region -Z4: 21,019,804-21,019,783	85 92 90 86 86	
	UM-F 5'-TTTGTTTTTTGGGTTTAAGT-3' Region -X: 21,062,242-21,062,223 Region -Z1: 21,040,007-21,039,988 Region -Z2: 21,029,725-21,029,706 Region -Z3: 21,023,907-21,023,888 Region -Z4: 21,019,908-21,019,889	95 95 100 100 95	
	UM-R5'-CCAACATAATAAAACCCCA-3' Region -X: 21,062,138-21,062,119 Region -Z1: 21,039,903-21,039,885 Region -Z2: 21,029,620-21,029,602 Region -Z3: 21,023,803-21,023,785 Region -Z4: 21,019,803-21,019,785	84 95 95 89 95	
Jiménez-Velasco <i>et al.</i> (2005)	M-F 5'-GTTTTAGATTCGAAAGGTCGTAGC-3' M-R 5'-AAAATAATTAACCCGTAACACGAT-3' UM-F 5'-GGTGTTTTAGATTTGAAAGGTTGTAGT-3' UM-R 5'-AAAAAACTAATTAACCCATAAAACAAT-3'	- - - -	(5)
Steinmann <i>et al.</i> (2009)	M-F 5'-TTCGTTCCGATTGGTATGCGGTC-3' M-R 5'-CCATCTTCCCGAAACGCTCACG-3' UM-F 5'-GGTGTTTTGTGGATTGGTATGTGGTT-3' UM-R 5'-CATCTTCCCAAACACTCACACCACA-3'	- - - -	(9)

M, methylated; UM, unmethylated; F; forward; R, reverse.

Another CpG island, region Y, located 333 bp downstream from the sequencing site, was also analyzed using the NGS-based methylation assay in eight BCC lines and two normal breast cell lines (Fig. 5A). No methylation was found in any of these cell

lines, a finding which is consistent with previous reports (4,9). Therefore, this CpG island does not appear to be involved in the epigenetic regulation of the *LATS2* gene. In addition, an NGS-based methylation assay was performed for region Z1,

which contained the most CpG sites among regions Z1-4, in eight BCC lines and two normal breast cell lines, and found that the methylation status of region Z1 was uniformly high in all cell lines, and its methylation status was not associated with the mRNA expression of *LATS2* (Fig. 5B). These results indicated that region Z1 was unlikely to be involved in the epigenetic regulation of the mRNA expression of *LATS2*.

In conclusion, the results obtained in the present study using NGS-based methylation assays demonstrated that *LATS2* hypermethylation was not involved in the silencing of the mRNA expression of *LATS2*. The mRNA expression of *LATS2* was lower in tumor cells, compared with normal epithelial cells, which suggested the possible involvement of the downregulation of *LATS2* mRNA in the pathogenesis of breast cancer. However, the mechanism underlying this down-regulation remains to be elucidated. The conflicting results previously reported for *LATS2* promoter methylation obtained using MSP assays are considered to be attributable to the low fidelity of the MSP assay.

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