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ORIGINAL ARTICLE

Promoter methylation assay of *SASH1* gene in breast cancer

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Summary

Purpose: *To analyze the relationship between the expression of SASH1 and its methylation level of SASH1 gene promoter in human breast cancer.*

Methods: *Expression levels of SASH1 were examined in breast cancer tissues and adjacent normal tissues with immunohistochemistry and with real time PCR (RT-PCR) methylation analysis was performed with MassArray.*

Results: *Immunohistochemistry showed that SASH1 expression was strongly reduced in breast cancer compared with adjacent normal tissues. Quantitative methylation analysis by MassArray revealed that CpG sites in SASH1 promoter shared similar methylation pattern in tumor tissue and adjacent normal tissue. The CpG sites with significant difference in methylation level were CpG_26.27 and CpG_54.55. Moreover, 5-aza-2'-deoxycytidine (5-Aza-dc) treatment of tumor cell line MDA-MB-231 caused significant elevation of SASH1 mRNA.*

Conclusion: *Based on these data, we propose that increase of DNA methylation level in the promoter region of gene SASH1, particularly CpG_26.27 or CpG_54.55 sites, possibly repressed SASH1 expression in breast cancer.*

Key words: *breast cancer, DNA methylation, SASH1, tumor suppressor gene*

Introduction

Cancer is the second leading cause of death in the United States and some European countries, such as Finland and Scotland [1-3]. The knowledge of molecular genetic mechanisms underlying tumorigenesis has increased since the discovery of tumor suppressor gene TP53 [4-9]. Tumor suppressor genes normally help prevent unrestrained cellular growth and promote DNA repair and cell cycle checkpoint activation. To date, many tumor suppressor genes have been found with germ-line mutation, such as ATM, CHK2, FOXO1, PTEN and NBS1 [8]. Among them, loss-of-function of TP53 and PTEN are associated with high risk for various cancers. However, not all loss-of-function of tumor suppressor genes are due to germ-line mutation [10]. For example, the epigenetic deregulation also contributes to the abnormal function of these genes [11,12]. Several members of the RASSF family of tumor suppressor genes are frequently epigenetically inactivated in cancer. RT-PCR analysis showed that loss or down-regulation of RASSF10 (one member of RASSF family) expression correlated with the methylation status of its CpG island in leukaemias [13].

SASH1, a novel tumor suppressor gene, mapped on 6q24.3, is possibly involved in tumorigenesis of breast and other solid tumors. It is a member of the SH3-domain containing expressed in lymphocytes (SLY1) gene family that encodes signal adapter proteins composed of several protein-protein interaction domains. The other members of this family are expressed mainly in haematopoietic cells, whereas SASH1 shows ubiquitous expression. It is down- regulated in the majority (74%) of breast tumors in comparison with corresponding normal breast epithelial

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tissues. Moreover, expression levels of SASH1 are strongly and significantly reduced in colon cancer of UICC stage II, III, and IV, as well as in liver metastases. However, no mutation has been found in the coding region of the gene in cancer tissues so far. Whether its expression is regulated through methylation is yet to be known [11,14].

In order to confirm whether methylation participated in the regulation of SASH1 gene expression in breast cancer, we investigated the methylation level of SASH1 gene promoter region and its expression level in breast cancer tissues. In addition, the impact of 5'-Aza-dC treatment on MDA-MB 231 cell line was also studied.

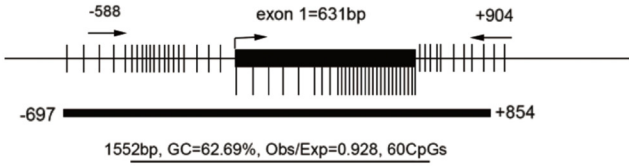

Figure 1. 5'end of SASH1 gene, indicating position of CpG islands and CpG sites used for DNA methylation analyses. Methylation analysis region is shown by inward facing arrows. The predicted transcriptional start site from the UC Santa Cruz Genome Browser is shown with bent right arrows, and exon is shown with black filled bar. Vertical stripes indicate CpG sites. Gray filled bar shows 5'CpG island; CpG island characteristics as determined using on-line EpiDesigner BETA software (http://www. epidesigner.com/) are shown beneath the gray bar.

Methods

Materials

Written informed consent regarding the use of the tissue samples was obtained from each subject before the study. Seventeen breast cancer samples (named as "breast tumor") and adjacent matched normal tissues (named as "breast nontumor") from breast cancer patients were collected from the Henan Cancer Hospital (Zhengzhou,China). All of the 17 breast cancer samples were utilized for MassArray quantitative methylation and immunohistochemical analysis. The study was performed after approval of the local ethics committee. The MDA-MB-231 cell line used in this study were obtained from the Shanghai cells bank of the Chinese Academy of Sciences (Shanghai, China).

DNA/RNA extraction

Genomic DNA was isolated using AxyPrep gDNA Isolation Mini Kit (Biosciences, Shanghai, China). RNA was extracted using Aqua-SPIN RNA Isolation Mini Kit (Watson Biotechnologies, Shanghai, China). The concentration and quality of the isolated DNA and RNA were measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA).

Bisulfite conversion and MassArray quantitative methylation analysis

Bisulfite treatment of genomic DNA was performed using the Ez DNA Bisulfite Treatment Kit (ZYMO Research, CA, USA) as recommended by the manufacturer. Quantitative methylation was measured using the MassArray Compact System, following the MassCLEAVE training protocol (Sequenom, San Diego, CA, USA) at CapitalBio Corporation (Beijing, China). The target CpG island in the promoter region is shown in Figure 1, and the primer pairs in Table 1. The resultant methylation calls were analyzed on EpiTyper software (Sequenom, CA, USA) to generate quantitative CpG methylation results.

Table 1. Primers used for MassArray quantitative methylation analysis

Genes	*Primers*	*Sequences (5'-3')*	*Length (bp)*
SASH1	meth2s	aggaagagagAGGGTTTTAAGTGTGTGTAGGTTGA	262
	meth2a	cagtaatacgactcactatagggagaaggctACCTCTACCAACCAAAACTTCTCTA	
	meth5s	aggaagagagAGAAGTTTTGGTTGGTAGAGGTAGG	392
	meth5a	cagtaatacgactcactatagggagaaggctCCTCTAAATCTTAAAAAAAACTCCCC	
	meth10s	aggaagagagGAGTTATGTGGAGTTGGAAGAGTTT	254
	meth10a	cagtaatacgactcactatagggagaaggctCAACAAAATAACAAAAAACAAAATCC	
	meth12s	aggaagagagTTTGTTTTTTGTTATTTTGTTGTTG	435
	meth12a	cagtaatacgactcactatagggagaaggctAAATAACTTACCAAAATACCCATCAC	
	meth14s	aggaagagagTATTTTGGTAAGTTATTTGGGGAGG	281
	meth14a	cagtaatacgactcactatagggagaaggctACACCAACATATCAACAAATATCCC	

Table 2. Primers used for real time PCR

Genes	*Primers*	*Sequences (5'-3')*	*Length (bp)*
SASH1	sashsense	TCGGCTTGACATTTGGACAG	175
	sashantis	CCTCTTCTGCTGCGACTTTC	
GAPDH	gapdhsense	CAAGAAGGTGGTGAAGCAGG	116
	gapdhantis	CGTCAAAGGTGGAGGAGTGG	

Promoter composition analysis

The putative transcription factor binding sites inside the SASH1 promoter were obtained using the TFSEARCH software (http://mbs.cbrc.jp/research/db/TFSEARCH.html) and WWW promoter scan software (http://www-bimas.cit.nih.gov/molbio/proscan/index.html).

Immunohistochemical analysis

The tumor samples were fixed in 4% phosphate buffered paraformaldehyde for 4 h or more and soaked into 30% sucrose solution for 3 days. Then the samples were embedded in OCT medium, sectioned to slides of 12 μm thickness, placed on specified glasses for immunohistochemistry, and stored at -80 ^{O}C ready for assay. The immunohistochemistry assay was performed according to the manufacturer's protocol (BOSTER BIO-engineering limited Company, Wuhan, China). Negative controls were prepared using the same procedure except that the primary antibodies (dilution1:400, Bethyl Laboratories Inc, USA) were replaced by antibody diluting solution. In order to compare the relative expression level between the tumor and the corresponding nontumor samples, 5 random fields were selected on the resultant images and their gray values were measured with Scion image software. The comparison was tested by independent sample T test with SPSS 15.0 software.

5-Aza-dC treatment

Human cell line MDA-MB-231 was incubated for 72 h with 50μ mol/L 5-aza-dC (Sigma -Aldrich, Germany) with a medium change every 24 h. RNA was isolated from treated cells as described above.

Real time PCR

First-strand cDNA was synthesized using PrimeScript RT reagent kit according to the manufacturer's instructions (Takara, Ostu, Shiga, Japan). The SASH1 gene was co-amplified with a fragment of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, which served as an internal standard. RT-PCR was conducted by the SYBR Premix Ex Taq kit (Takara, Ostu, Shiga, Japan) on the ABI 7900HT Fast Real-Time PCR System (Life Technologies, CA, USA). The primer pairs are shown in Table 2 and the cycling conditions of 40 cycles of PCR were 95 ^{O}C/5 sec, 55 ^{O}C/30 sec, and 72 ^{O}C/30 sec. Each sample was run in 4 repeats and all the PCR data were analyzed with the ABI 7900HT system software 2.3 version.

Table 3. Different CpG sites and putative transcription binding factor

Difference CpG sites	*Sequence*	*Transcription factor*
CpG-4	TTGGGTCGCTTGA	none
CpG-8.9	CTCGGGTTTCCGT	none
CpG-14	CCTGTCCGAGGCT	none
CpG-17	GGTCCCGGGGAGC	none
CpG-26.27	CCCCGCCGTACAA	SP1/MZF-1
CpG-28	GTGGTGCGGACTT	none
CpG-34.35.36	CGGGCGCCTGCGA	none
CpG-38	GACACGGCCATGG	none
CpG-43	CTGAGCCCGAGCC	none
CpG-44	CCCGAGCCGGAGC	none
CpG-46	GAGCCCGAGCCCG	none
CpG-51.52	GGCGTTCTCCCGAC	none
CpG-54.55	GCGGCGCAGGAAG	Elk-1
CpG-57.58	CGGGGGCTCCCTTCTCG	none
CpG-59	CAGAGGCGTCCTT	none
CpG-60	TCCCGTCAAAAGG	none

Statistics

The methylation rates in two independent sample groups were compared using Mann-Whitney test. RT-PCR results were compared using independent sample T test between two groups. All p-values were two-sided and $p < 0.05$ was considered statistically significant. The SPSS version 15.0 (SPSS Inc, Chicago, IL, USA) was used for all statistical analyses. The cases hierarchical cluster analysis clustered the 60 CpG sites in the SASH1 promoter based on Euclidean distances and the average linkage clustering algorithm. This clustering was implemented using Cluster 3.0 and viewed on Java Treeview.

Results

DNA methylation status of SASH1 gene promoter in breast cancer

According to MassArray quantitative methylation analysis in 17 breast cancer samples, the mean methylation level of each CpG site was used to be compared between nontumor and tumor tissues (Figure 2). Significant differences ($p<0.05$) were revealed at the following CpG sites: CpG_4, CpG_8.9, CpG_14, CpG_17, CpG_26.27, CpG_28,

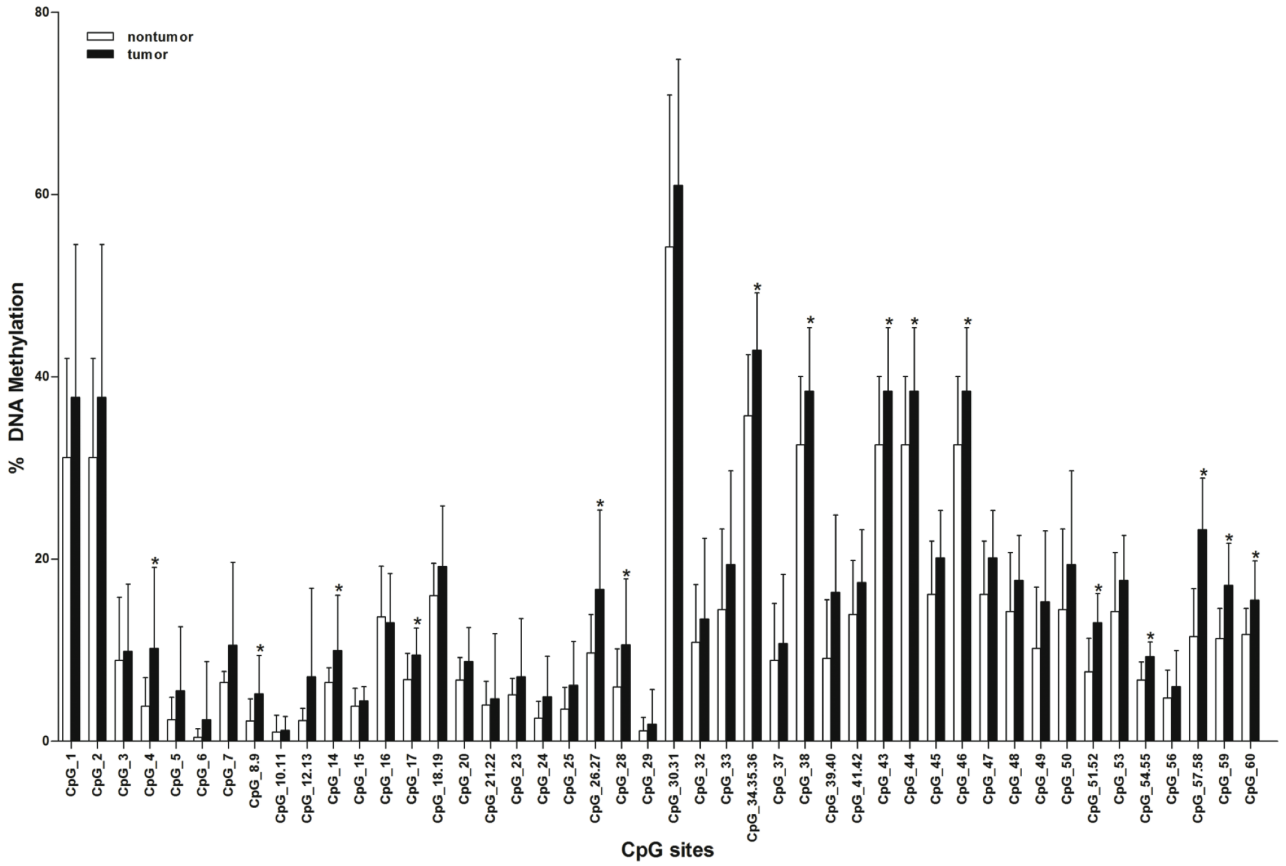

Figure 2. Comparison of mean methylation for each CpG site between nontumor and tumor. The X-axis represents 60 informative CpG sites within 5 MassArray amplicons for the SASH1 promoter; the Y-axis shows the average methylation value of each CpG site (or clusters of CpG sites). Error bars =SD. Significant differences are indicated by* ($p<0.05$).

CpG34.35.36, CpG_38, CpG_43, CpG_44, CpG_46, CpG_51.52, CpG_54.55, CpG_57.58, CpG_59 and CpG-60.

Promoter composition analysis with WWW promoter scan software revealed that the promoter region was 330 nucleotides in length within exon-1 and 33 binding sites for transcription factors were yielded in this region. Querying the same sequence (the methylation analysis region) with TFSEARCH software displayed 96 binding sites for transcription factors covering the whole sequence. Together with the difference CpG sites, only CpG_26.27 and CpG_54.55 sites possessed binding sites for transcription factors such as MZF1, SP1 and Elk-1 (Table 3). Therefore, we speculated that the site CpG_26.27 or CpG_54.55 were perhaps correlated with the SASH1 gene expression.

Then, the general methylation feature across the whole promoter region was analyzed. The mean methylation range of different CpG sites was from 1.21% (at CpG_10.11) to 61.00% (at CpG _30.31) in breast tumor tissues and from 0.43% (at CpG_6) to 54.25% (at CpG _30.31) in breast nontumor tissues. After unsupervised clustering, it was shown that different CpG sites in SASH1 promoter shared similar methylation pattern, namely different CpG sites simultaneously had high or low methylation levels in breast tumor and nontumor tissues (Figure 3A). However, with clustering of the ratios of methylation level (tumor/nontumor), the methylation levels were up-regulated at most CpG sites (represented in red in Figure 3B) compared with nontumor tissues (Figure 3B). The mean methylation level of breast tumor was 16.80% and that of breast nontumor 12.86%. Although the mean methylation level was increased in tumor tissues as compared to adjacent normal tissues, there was no significant difference between them.

Down-regulation of SASH1 in tumor tissues vs nontumor tissues and up-regulation after restoration experiments with 5-aza- dC

In SASH1 immunohistochemistry of 17 breast samples, SASH1 was mainly expressed in the mammary glands (indicated by the arrow at the right side of Figure 4A) in the nontumor group.

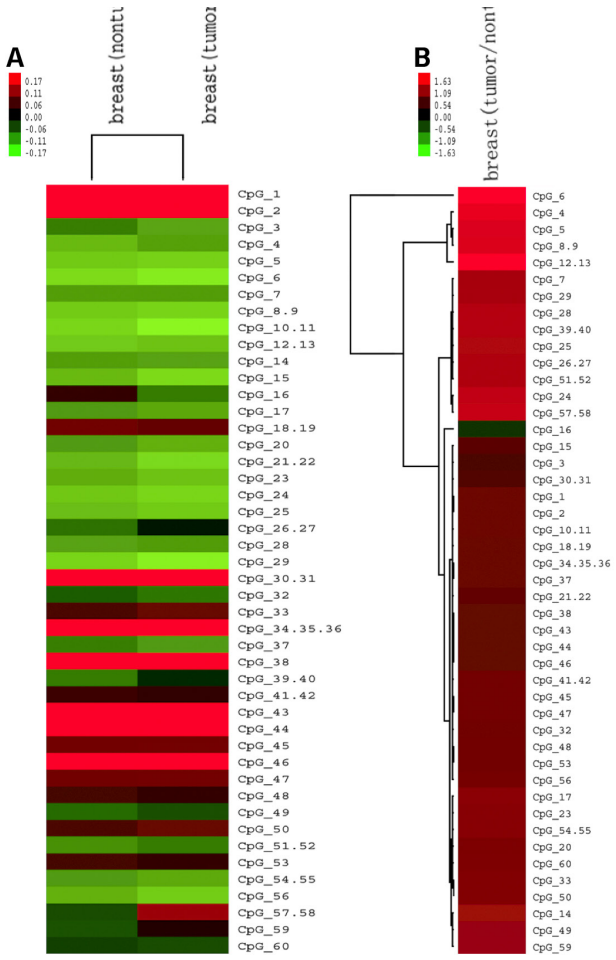

Figure 3. MassArray quantitative methylation of the SASH1 promoter. **(A)**: The hierarchical cluster analysis of methylation patterns of 60 CpG sites measured on 17 samples. The methylation level (subtracting the general mean value) of each CpG site within each sample is presented in the plot with color ranging from green (indicating low methylation) to red (indicating high methylation). **(B)**: The hierarchical cluster analysis of methylation patterns of 60 CpG sites measured on samples as above. The methylation level (log2 ratio tumor/nontumor) of each CpG site within each sample is presented in the plot with color ranging from green (indicating methylation level of tumor lower than that of nontumor) to red (indicating methylation level of tumor higher than that of nontumor).

However, the tumor group displayed little protein expression (indicated as above).The relative expression of SASH1 represented with gray value was applied to statistical analysis and significant difference ($p<0.0001$) was observed between nontumor and tumor group (Figure 4A).

To verify the functional relationship between promoter methylation increase and loss of SASH1 gene expression, mRNA expression levels were compared before and after treatment with 5-aza-dC in MDA-MB-231 cell line. The expression increased about 4-fold after treatment. These data suggested that methylation up-regulation of SASH1 promoter repressed SASH1 expression.

Discussion

It has previously been demonstrated that reduced expression of SASH1 may not be attributed to somatic mutations in the coding sequences of SASH1.Other mechanisms are likely responsible for the loss of expression of SASH1 in breast cancer [11]. In light of this, we performed MassArray quantitative methylation analysis in the promoter CpG island of SASH1 in breast cancer. Our results indicated that tumor and nontumor tissues tended to share the common methylation pattern at different CpG sites (Figure 3A). However, composition analysis revealed that only partial CpG sites possessed binding sites for transcription factors. These suggested that different CpG sites had different functions or only partial CpG sites were crucial for regulation in gene expression. Regarding DNA methylation, the low level of CpG methylation in tumors compared with their normal tissue counterparts was one of the first epigenetic alterations to be found in human cancer. According to this phenomenon, we could see that not methylation itself but the change in methyla-

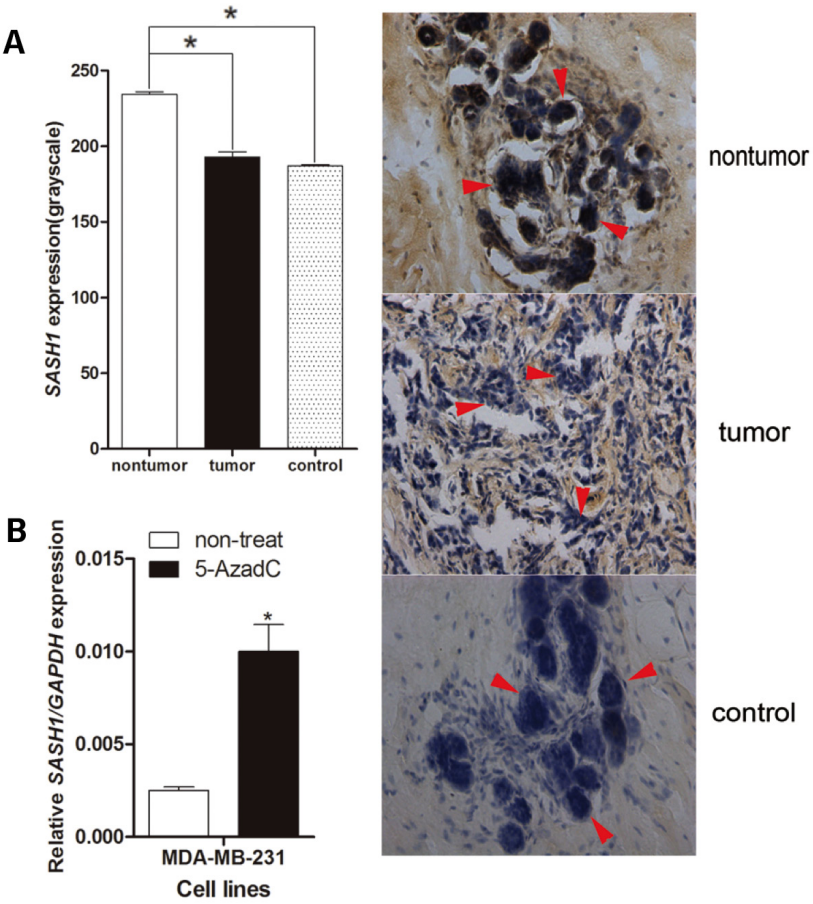

Figure 4. SASH1 expression analysis. **(A)**: Immunohistochemistry analysis of SASH1 expression in breast tissues. The bar graphs show gene expression levels by the gray value after Scion image software measure. Error bars =SD. Significant differences are indicated by* ($p<0.001$). Immunohistochemistry results are shown on the right side of the Figure. Arrows indicate the reaction region. **(B)**: Quantitative analysis of SASH1 mRNA in cells before and after treatment with 5-AzadC. The results are expressed as the ratio of copies of target gene relative to GAPDH. Error bars=SD. Significant differences are indicated by* ($p<0.001$).

tion was more important in contributing to tumorigenesis [15]. In our study, after comparison between tumor tissues and adjacent normal tissues at different CpG sites, many CpG sites with statistical significance in the methylation level ($p<0.05$) were found, such as CpG_26.27 and CpG_54.55. Therefore, we speculated that individual CpG site might be more important in gene expression regulation.

To investigate the correlation between SASH1 methylation variation and protein expression, the immunohistochemistry was performed on 17 patient samples. The results exhibited nominally a significant decrease in SASH1 expression ($p<0.05$) in tumor tissues as compared to adjacent normal tissues. Our findings were in agreement with observations in colon cancer or breast cancer of patients in Germany [11,14]. Thus, promoter methylation discrepancy might be the cause for the significant decrease of SASH1 protein. This concept was further supported by the fact that the SASH1 expression was restored after the cultured MDA-MB-231 cells were treated with 5-aza-dC.

Our results substantiated that the methylation alteration was involved in gene SASH1 expression. But we could not exclude the possibility that other epigenetic mechanisms, for example histone deacetylation or microRNA also contributed to SASH1 gene down-regulation [16]. The SASH1 CpG island in promoter was subjected to promoter scan and TFSEARCH softwares to generate a transcription regulation map [17]. Together with the significant difference in CpG sites analysis, we found the higher methylation might block the interaction between the CpG_26.27 site or CpG_54.55 site of SASH1 promoter and one of transcription factors as follows: SP1, MZF1 and Elk-1.

Sp1 is the prototypic member of the Sp/ Krüppel-like family of zinc-finger proteins that function

as transcription factors in mammalian cells [18]. MZF1, a transcription factor belonging to the Kruppel family of zinc-finger proteins, was reported to be a bifunctional transcription regulator [19]. Elk-1 , a transcription factor, is a member of the ETS subfamily HCF and it is an efficient substrate for all three classes of MAP kinase [20]. All these three transcription factors have been shown to regulate many genes and take part in virtually all facets of cellular function, including cell proliferation, apoptosis, and differentiation. Besides the functions in physiological processes, they also have crucial roles in pathological processes such as tumorigenesis and genetic diseases [18,19,21-23]. At the global level, DNA is often hypo-methylated in cancer, but local hyper-methylation of individual genes is often associated with aberrant gene silencing, such as tumor suppressor genes [15]. The presence of binding sites of SP1, MZF1 or Elk-1 in the SASH1 promoter suggested that inhibition of SASH1 expression might be accounted for by the decrease in binding affinity between one of these transcription factors and SASH1 promoter due to the increased methylation level in cancer. Of course, we could not exclude the possibility that other mechanisms might be involved in the inhibition of SASH1 expression. For example, other transcription factors might be required for a coordinate in the regulation of SASH1 expression. Similarly, we could not rule out that other different CpG sites might also take part in the regulation of SASH1 expression. The exact mechanism still remains unclear and thus further investigations are warranted.

In conclusion, the present study indicated that methylation up-regulation at CpG_26.27 or CpG-54.55 in SASH1 promoter might be involved in SASH1 gene expression inhibition through blocking the interaction between the SASH1 promoter and one of the three transcription factors (MZF1, SP1and Elk-1) (Table 3).

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