

ORIGINAL ARTICLE

Promoter methylation assay of SASH1 gene in hepatocellular carcinoma

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Summary

Purpose: To analyse the relationship between the expression of SASH1 and its methylation level in human hepatocellular carcinoma. **Methods:** Expression levels of SASH1 were examined with real-time PCR (RT-PCR) in tissues and cells, and methylation analysis was performed with MassArray.

Results: The expression levels of SASH1 were strongly reduced in liver cancer tissues compared with adjacent normal tissues. Quantitative methylation analysis by MassArray revealed different CpG sites in SASH1 promoter shared similar methylation pattern between liver cancer tissues and adjacent normal tissues and the CpG sites of significant

difference in methylation level were found as follows: CpG_3, CpG_17, CpG_21.22, CpG_25, CpG_26.27, CpG_28, CpG_34.35.36 and CpG_51.52. Moreover, 5-aza-2'-deoxycytidine treatment of Hep-G2 cell line caused significant elevation of SASH1 mRNA.

Conclusions: Based on these data, we propose that increase of DNA methylation degree in the promoter region of SASH1 gene, particularly CpG_26.27 sites, possibly repressed SASH1 expression in liver cancer.

Key words: DNA methylation, SASH1, tumor suppressor gene

Introduction

Cancer is the second leading cause of human deaths 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, epigenetic deregulation also contributes to 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 the 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 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 in hepatocellular carcinoma is yet to be known [11,14].

To examine the methylation profiles of 60 CpG sites in the SASH1 promoter in liver cancer tissues, we performed a MassArray methylation analysis. Several CpG sites of significant differences in methylation level between tumor tissues and adjacent normal tissues were found. At the same time, the expression of SASH1 was determined by RT-PCR and restoration experiments performed with 5-aza-2'-deoxycytidine drastically increased SASH1 mRNA levels in HepG2 tumor cell line. Taken together, our study suggested that methylation alteration in SASH1 promoter was possibly involved in SASH1 expression.

Methods

Materials

Informed, written consent regarding the use of the tissue samples was obtained from each subject before the study. Forty three hepatocellular carcinoma specimens [named as liver (tumor)] and matched adjacent normal tissues [named as liver (nontumor)] from liver cancer patients were collected from the Affiliated Hospital of Nantong University (Nantong, China). In total, 43 hepatocellular carcinoma samples were utilized for MassArray quantitative methylation analysis and RT-PCR. The study was performed after approval of the local ethics committee. The Hep G2 cell lines used in this study were obtained from the Shanghai Cells Bank of 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.

Promoter composition analysis

The putative transcription factor binding sites inside the SASH1 promoter were obtained using the WWW promoter scan software (<http://www.bimas.nih.gov/molbio/proscan/index.html>).

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. Q-PCR was conducted by the SYBR Premix Ex Taq kit (Takara, Ostu, Shiga, Japan) on the ABI 7900HT Fast RT-PCR System (Life Technologies, CA, USA). The primer pairs showed in Table 2 and the cycling conditions of 40 cycles of PCR

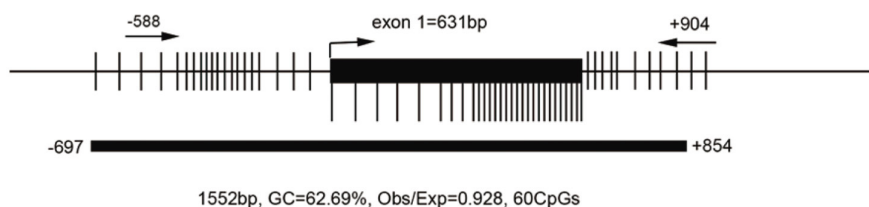


Figure 1. 5' end of SASH1 gene, indicating the position of CpG islands and CpG sites used for DNA methylation analyses. Methylation analysis region is shown by inward facing arrows. The predicted transcriptional start site from the UC Santa Cruz Genome Browser is shown with bent right arrows, and exon 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.

Table 1. Primers used for MassArray quantitative methylation analysis

SASH1	meth2s	aggaagagagAGGGTTTAAAGTGTGTGTAGGTTGA	262
	meth2a	cagtaatacactcactataggagaaggctACCTCTACCAACCAAACTTCTCTA	
	meth5s	aggaagagagAGAAGTTTGGTTGGTAGAGGTAGG	
	meth5a	cagtaatacactcactataggagaaggctCCTCTAAATCTTAAAAAACTCCCC	392
	meth10s	aggaagagagGAGTTATGTGGAGTTGGAAGAGTTT	
	meth10a	cagtaatacactcactataggagaaggctCAACAAAATAACAAAAACAAAATCC	
	meth12s	aggaagagagTTTGTTTTTTTATTGTTGTTG	435
	meth12a	cagtaatacactcactataggagaaggctAAATAACTTACCAAAATACCCATCAC	
	meth14s	aggaagagagTATTTTGGTAAGTTATTTGGGGAGG	
	meth14a	cagtaatacactcactataggagaaggctACACCAACATATCAACAAATATCCC	281

Table 2. Primers used for RT-PCR

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

Table 3. CpG sites with differences and putative transcription binding factors

Difference CpG sites	Sequence	Transcriptional factors
CpG-3	TAGCCGAAAGCCC	none
CpG-17	GGTCCCGGGGAGC	none
CpG-21.22	GTCGCGTGCCCTTA	none
CpG-25	CTTCTGGGCCGAG	none
CpG-26.27	CCCCGCCGTACAA	SP1/MZF-1
CpG-28	GTGGTGCGGACTT	none
CpG-34.35.36	CGGGCGCCTGCGA	none
CpG-51.52	GGCGTTCTCCCGAC	none

were 95°C/5 s, 55°C/30 s, and 72°C/30 s. Each sample was run in 4 repeats and all the PCR data were analyzed with the ABI 7900HT system software 2.3 version.

5-Aza-dC treatment

Human Hep G2 cell line was incubated for 72 hrs with 50μ mol/L 5-aza-dC (Sigma- Aldrich, Germany) with a medium change every 24 hrs. RNA was isolated from treated cells as described above.

Statistics

The methylation rates in two independent sample groups were compared using Mann-Whitney U test. RT-PCR results were compared using independent sample T test between two groups. All p-values were two-sided, p <0.05 was considered statistically significant. 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 liver cancer

According to MassArray quantitative methylation analysis in 43 hepatocellular carcinoma 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_3, CpG_17, CpG_21.22, CpG_25, CpG_26.27, CpG_28, CpG_34.35.36 and CpG_51.52.

Promoter composition analysis with WWW promoter scan software revealed that the promoter region of 330 nucleotides in length including exon-1 possessed 33 binding sites for transcription factors. 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 of CpG sites, only CpG_26.27 site possessed binding sites for transcription factors such as MZF1 and SP1 (Table 3). Therefore, we speculated that the CpG_26.27 site was perhaps

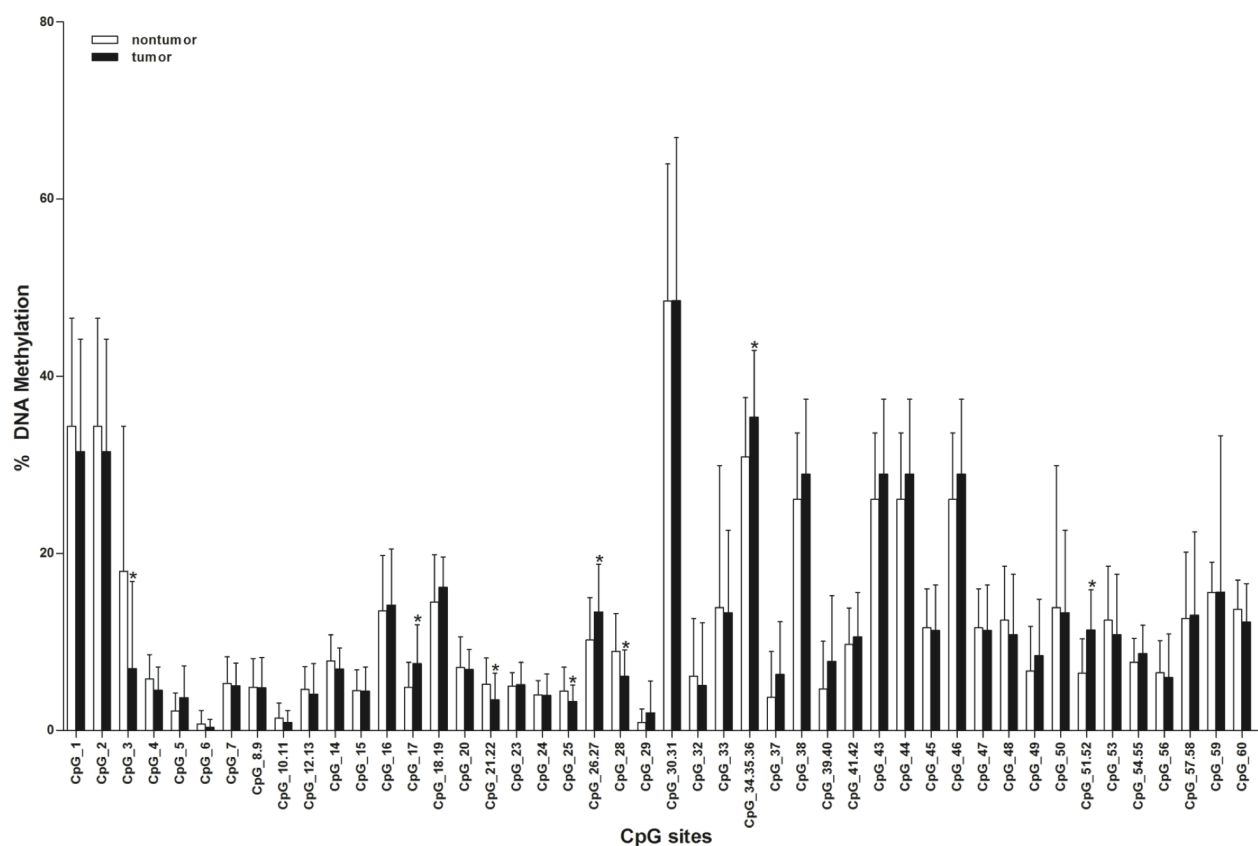


Figure 2. Comparison of mean methylation for each CpG site between nontumor and tumor. The X-axis represents 60 informative CpG sites within 5 MassArray amplicons for the SASH1 promoter; the Y-axis shows the average methylation value of each CpG site (or clusters of CpG sites). Error bars =SD. Significant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

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 0.38% (at CpG_6) to 48.52% (at CpG_30.31) in liver tumor tissues and from 0.72% (at CpG_6) to 48.5% (at CpG_30.31) in liver nontumor tissues. After unsupervised clustering, it was seen that different CpG sites in SASH1 promoter shared similar methylation pattern, namely, different CpG sites simultaneously had high or low methylation levels in tumor and nontumor tissues (Figure 3A). However, with clustering of ratios of methylation level (tumor:nontumor) in liver tissues, only partial CpG sites in methylation levels of tumor tissues were up-regulated (represented in red in Figure3B) compared with nontumor tissues (Figure 3B). The mean methylation level of liver tumor was 12.22% and that of liver nontumor was 12.07%. Although the mean methylation level was increased in tumor tissues as compared to that of adjacent normal tissues, there was no significant difference between them. Together

with the above analysis of individual CpG site, we could see that individual CpG site might be more important in gene expression regulation.

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

Using RT-PCR in 43 hepatocellular samples, SASH1 mRNA expression in the liver was quantified. As shown in Figure 4A, mRNA level in the tumor group decreased by about one-fold as compared to the nontumor group (mean ratio of tumor group=0.017, mean ratio of nontumor group=0.032, $p < 0.001$).

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 Hep-G2 cell line (Figure 4B). The expression increased up to 70% after treatment (mean ratio before treatment $p = 0.0013$, mean ratio after treatment $p < 0.0021$). These data suggested that

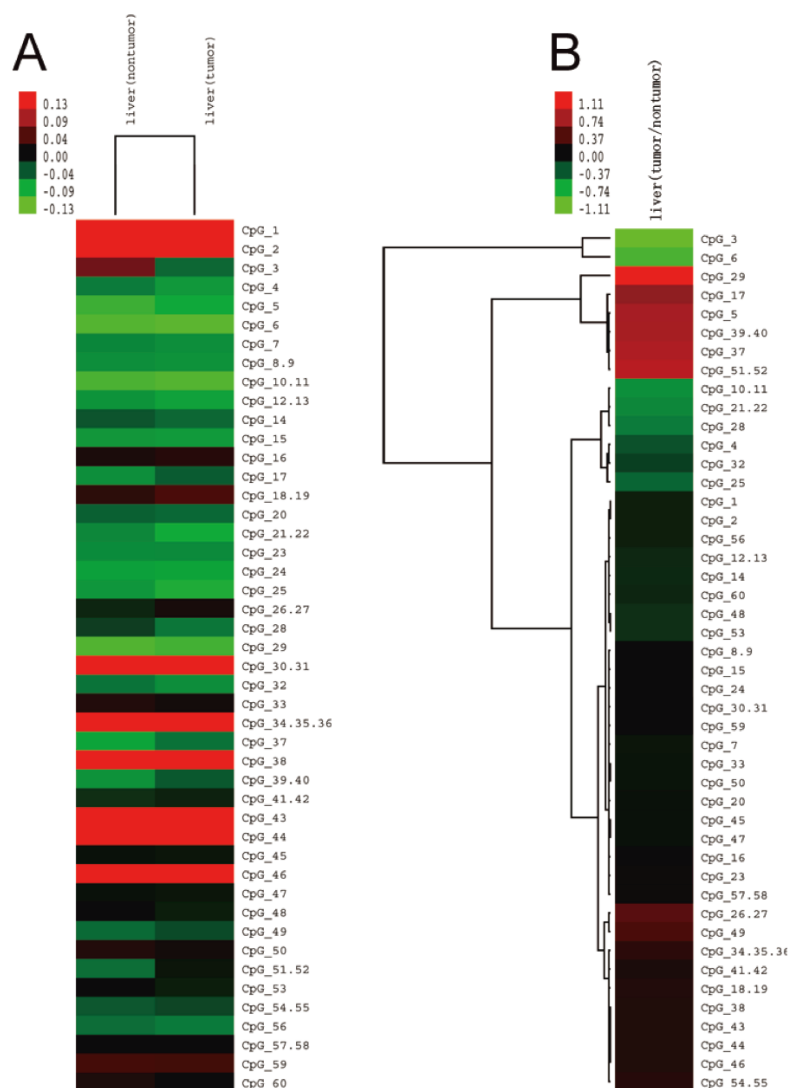


Figure 3. MassArray quantitative methylation of the SASH1 promoter. **(A)** Hierarchical cluster analysis of methylation patterns of 60 CpG sites measured on 43 samples. The methylation level (subtracting the general mean value) of each CpG site within each sample is presented in the plot with color ranging from green (indicating low methylation) to red (indicating high methylation). **(B)** Hierarchical cluster analysis of methylation patterns of 60 CpG sites measured on samples as above. The methylation level ($\log_2$ 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).

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 [11]. In light of this, we performed MassArray quantitative methylation analysis in the promoter CpG island of SASH1 in liver cancer. Our results indicated that tumor tissues and nontumor tissues

tended to share common methylation pattern at different CpG sites (Figure 3A). However, different CpG sites had different change pattern after tumorigenesis. 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 that in 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 methylation was more important in contributing to tumorigenesis [15]. In our study, after compar-

ison between tumor tissues and adjacent normal tissues at different CpG sites, many statistically significant CpG sites in methylation level ($p < 0.05$) were found such as CpG_3, CpG_17, CpG_21.22, CpG_25, CpG_26.27, CpG_28, CpG_34.35.36 and CpG_51.52.

To investigate the correlation between SASH1 methylation variation and mRNA expression, RT-PCR was conducted on a cohort of 43 patients. The results exhibited nominally 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, methylation discrepancy in promoter might be the cause for the significant decrease of SASH1 protein or mRNA. This concept was further supported by the fact that the SASH1 expression was restored after the cultured cells were treated with 5-aza-dC.

Our results substantiated that methylation alteration was involved in gene SASH1 expression. However, 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

er with CpG sites with differences in methylation level, we found higher methylation might block the interaction between the CpG_26.27 of SASH1 promoter and one of the transcription factors as follows: SP1 or MZF-1.

Sp1 is the prototypic member of the Sp/Kruppel-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]. These two 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 [18-22]. 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 in tumor suppressor genes [15]. The presence of binding sites of MZF-1 or SP1 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

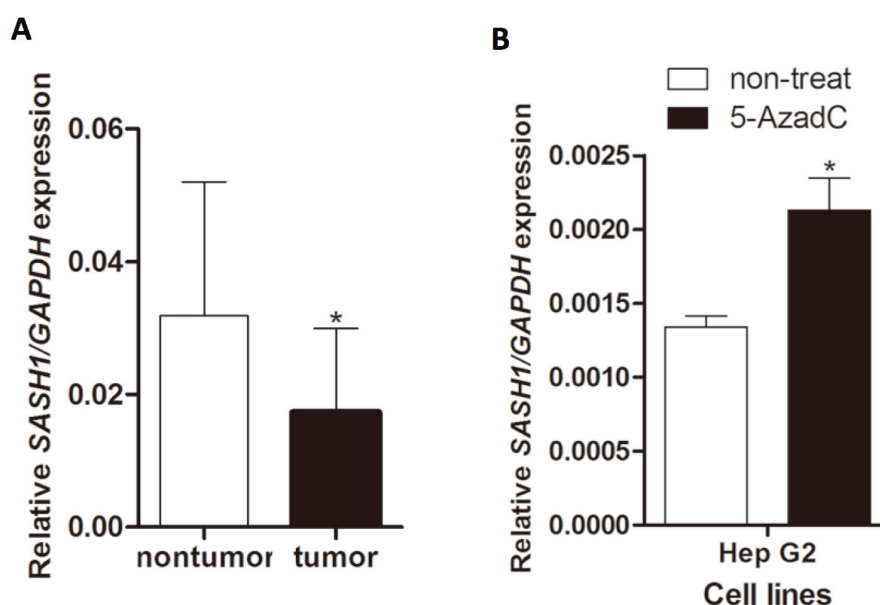


Figure 4. SASH1 expression analysis. **(A)** RT-PCR analysis of SASH1 expression in liver tissues. The bar graphs shows gene expression levels by the SASH1/GAPDH ratio. Error bars =SD. Significant differences were indicated by * ($p < 0.001$). **(B)** RT-PCR analysis of SASH1 mRNA in cells before and 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 * ($p < 0.001$).

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 other CpG sites might also take part in the regulation of SASH1 expression. However, 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 other significant different CpG_sites in SASH1 promoter might be involved in SASH1 gene inhibition of expression through blocking the interaction between the SASH1 promoter and one of the two transcription factors (MZF-1 and SP1) (Table 3).

References

- Villanueva A, Llovet JM. Targeted therapies for hepatocellular carcinoma. *Gastroenterology* 2011;140:1410-1426.
- Jousilahti P, Salomaa V, Kuulasmaa K, Niemela M, Vartiainen E. Total and cause specific mortality among participants and non-participants of population based health surveys: a comprehensive follow up of 54 372 Finnish men and women. *J Epidemiol Community Health* 2005;59:310-315.
- Gray L, Batty GD, Craig P et al. Cohort profile: the Scottish health surveys cohort: linkage of study participants to routinely collected records for mortality, hospital discharge, cancer and offspring birth characteristics in three nationwide studies. *Int J Epidemiol* 2010;39:345-350.
- Veeck J, Niederacher D, An H et al. Aberrant methylation of the Wnt antagonist SFRP1 in breast cancer is associated with unfavourable prognosis. *Oncogene* 2006;25:3479-3488.
- Beaumont T, Leadbeater M. Treatment and care of patients with metastatic breast cancer. *Nurs Stand* 2011;25:49-56.
- Dai C, Gu W. p53 post-translational modification: deregulated in tumorigenesis. *Trends Mol Med* 2010;16:528-536.
- Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res* 2002;62:1008-1013.
- Zhang H, Pan Y, Zheng L et al. FOXO1 inhibits Runx2 transcriptional activity and prostate cancer cell migration and invasion. *Cancer Res* 2011;71:3257-3267.
- Zhao Y, Kong X, Li X et al. Metadherin mediates lipopolysaccharide-induced migration and invasion of breast cancer cells. *PLoS One* 2011;6:e29363.
- Narayan G, Freddy AJ, Xie D et al. Promoter methylation-mediated inactivation of PCDH10 in acute lymphoblastic leukemia contributes to chemotherapy resistance. *Genes Chromosomes Cancer* 2011;50:1043-1053.
- Zeller C, Hinzmann B, Seitz S et al. SASH1: a candidate tumor suppressor gene on chromosome 6q24.3 is downregulated in breast cancer. *Oncogene* 2003;22:2972-2983.
- Lee EY, Muller WJ. Oncogenes and tumor suppressor genes. *Cold Spring Harb Perspect Biol* 2010;2:a003236.
- Hesson LB, Dunwell TL, Cooper WN et al. The novel RASSF6 and RASSF10 candidate tumour suppressor genes are frequently epigenetically inactivated in childhood leukaemias. *Mol Cancer* 2009;8:42.
- Rimkus C, Martini M, Friederichs J et al. Prognostic significance of downregulated expression of the candidate tumour suppressor gene SASH1 in colon cancer. *Br J Cancer* 2006;95:1419-1423.
- Berdasco M, Esteller M. Aberrant epigenetic landscape in cancer: how cellular identity goes awry. *Dev Cell* 2010;19:698-711.
- Goeppert B, Schmezer P, Dutruel C et al. Down-regulation of tumor suppressor A kinase anchor protein 12 in human hepatocarcinogenesis by epigenetic mechanisms. *Hepatology* 2010;52:2023-2033.
- Banerjee D, Nandagopal K. Potential interaction between the GARS-AIRS-GART Gene and CP2/LBP-1c/LSF transcription factor in Down syndrome-related Alzheimer disease. *Cell Mol Neurobiol* 2007;27:1117-1126.
- Dong S, Ying S, Kojima T et al. Crucial roles of MZF1 and Sp1 in the transcriptional regulation of the peptidylarginine deiminase type I gene (PADI1) in human keratinocytes. *J Invest Dermatol* 2008;128:549-557.
- Luo X, Zhang X, Shao W, Yin Y, Zhou J. Crucial roles of MZF-1 in the transcriptional regulation of apomorphine-induced modulation of FGF-2 expression in astrocytic cultures. *J Neurochem* 2009;108:952-961.
- Convertini P, Infantino V, Bisaccia F, Palmieri F, Iacobazzi V. Role of FOXA and Sp1 in mitochondrial acylcarnitine carrier gene expression in different cell lines. *Biochem Biophys Res Commun* 2011;404:376-381.
- Kim HJ, Ko MS, Kim HK et al. Transcription factor Sp1 regulates basal transcription of the human DRG2 gene. *Biochim Biophys Acta* 2011;1809:184-190.
- Gao SY, Li EM, Cui L et al. Sp1 and AP-1 regulate expression of the human gene VIL2 in esophageal carcinoma cells. *J Biol Chem* 2009;284:7995-8004.