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Gene silencing of SLC5A8 identified by genome-wide methylation profiling in lung cancer

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Abstract

Background—Aberrant DNA hypermethylation has been implicated as a component of an epigenetic mechanism that silences genes in cancers.

Methods—We performed a genome-wide search to identify differentially methylated loci between 26 tumor and adjacent non-tumor paired tissues from same lung cancer patients using restriction landmark genomic scanning (RLGS) analysis. Among 229 loci which were hypermethylated in lung tumors as compared to adjacent non-tumor tissues, solute carrier family 5, member 8 (SLC5A8) was one of the hypermethylated genes, and known as a tumor suppressor gene which is silenced by epigenetic changes in various tumors. We investigated the significance of DNA methylation in SLC5A8 expression in lung cancer cell lines, and 23 paired tumor and adjacent non-tumor lung tissues by reverse transcription-PCR (RT-PCR), quantitative methylation specific PCR (QMSP) and bisulfite modified DNA sequencing analyses.

Results—Reduced or lost expression of SLC5A8 was observed in 39.1% (9/23) of the tumor tissues as compared with paired adjacent non-tumor tissues. Bisulfite sequencing results of lung cancer cell lines and tissues which did not express SLC5A8 showed a densely methylated promoter region of SLC5A8. SLC5A8 was reactivated by treatment with DNA methyltransferase

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Conflicts of interest statement

The authors declare that they have no conflict of interest.

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inhibitor, 5-Aza and/or HDAC inhibitor, trichostatin A (TSA) in lung cancer cell lines, which did not express SLC5A8. Hypermethylation was detected at the promoter region of SLC5A8 in primary lung tumor tissues as compared with adjacent non-tumor tissues (14/23, 60.9%).

Conclusion—These results suggest that DNA methylation in the SLC5A8 promoter region may suppress the expression of SLC5A8 in lung tumor.

Keywords

Lung cancer; Gene silencing; SLC5A8; Tumor suppressor gene; DNA methylation; Restriction landmark genomic scanning

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in the United States. The poor survival is due to its advanced stage at the time of diagnosis. Over 50% of patients were found to have distant metastatic lesion at the time of diagnosis and only 15% of patients had localized disease of whom the survival could be expected 5 years with potentially-curative resection [1]. Recently, the National Lung Screening Trial has shown low-dose helical computed tomography to reduce lung cancer mortality [2], however there are still uncertainty to screen general population.

DNA methylation of promoter regions in tumor suppressor genes is considered as one of the contributing factors in carcinogenesis. The methylation status of the promoter region in many genes has been assessed in lung tumors. Several genes are known to be hypermethylated and are reported to be of unfavorable prognostic relevance in primary lung tumor [3]. Furthermore, promoter methylations in these genes were found in the early stage of carcinogenesis and remained during progression [4].

Solute carrier family 5 (iodide transporter) (SLC5) is a solute-linked carrier gene family that contains 12 sodium-coupled transporters for several chemicals [5]. Several studies reported that SLC5A8 is a sodium-coupled transporter for nicotinate and its structural analogs [6], lactate [7], pyroglutamate [8], short-chain fatty acids (butyrate) [9] and pyruvate which are known as histone deacetylase (HDAC) inhibitors [10–14].

SLC5A8 was first identified as a differentially methylated gene by restriction landmark genome scanning (RLGS) which provides a global analysis of methylation events in colon cancer cell lines [15]. Since then, increasing evidences suggest that gene silencing of SLC5A8 may contribute to the carcinogenesis and progression of tumors. Therefore, SLC5A8 has been suggested as a tumor suppressor and promoter methylation and gene-silencing in various tumors has been detected [4, 15–27].

In the present study, we report a differential methylation profile of lung tumor tissues, characterize the methylation status of SLC5A8 and investigate the role of epigenetic changes in the promoter region of SLC5A8 in its expression in lung cancer cell lines and lung tumor tissues.

MATERIALS AND METHODS

Tissue collection

Paired lung tumor and adjacent non-tumor tissues from 26 lung cancer patients were used for RLGS analysis. These patients were operated at the Moffitt Cancer Center (Tampa, FL) with primary adenocarcinoma (n=14), squamous cell carcinoma (n=9) and large cell carcinoma (n=3). The patients included 25 Caucasians and 1 African American, 18 males

and 8 females ranging in age from 51 to 87 years (Table 1). For expression and DNA methylation analyses, 23 pairs of lung tissues which were analyzed in RLGS assay, were used. Three paired lung tumor and adjacent non-tumor tissues were excluded, because there were not enough samples left for analysis.

All tissues were collected under a protocol approved by the Institutional Review Board of the Moffitt Cancer Center and the University of South Florida. All adjacent non-tumor specimens were harvested freshly from morphologically normal appearing tissue located at least 3 cm from the tumor margin. A surgical pathologist performed histological evaluation of the tumor and non-tumor tissues. All tissues were snap frozen in liquid nitrogen and stored at -80°C .

Restriction landmark genome scanning (RLGS) analysis

After extracting DNA from the lung tissues, the RLGS technique, a two-dimensional gel electrophoresis system that uses radiolabeled restriction endonuclease sites to create “landmarks” seen on an autoradiograph, was used as previously described [28–30]. The tumor and normal gels of each patient were either visually compared or quantified by densitometry at each of ~2,000 sites. Differential methylation was detected by either the absence or decrease in signal intensity ($> 50\%$).

RNA extraction from cell lines and tissues

RNA was extracted from 22 lung cancer cell lines, which were obtained from the American Type Culture Collection (Manassas, VA), and 23 paired lung tumors/adjacent non-tumors tissues with the Qiagen RNA isolation kit (Austin, TX) according to the manufacturer’s protocol.

Semi-quantitative and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

We investigated the expression of SLC5A8 in lung cancer cell lines, and paired tumor and adjacent non-tumor tissues from lung cancer patients by a semi-quantitative and quantitative real-time RT-PCR. Semi-quantitative PCR was performed with primers selected from exon 1 and exon 2 (cttcattgctcagcgcctactg and caatgaagaggactgtccac, respectively). The resulting amplified DNA was analyzed by electrophoresis on an 8% PAGE gel as previously described [22].

Quantitative real-time PCR was performed using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol. Taqman Gene Expression Assay including 2 primers and a probe was used for the quantification of SLC5A8, and β -actin was used as reference gene as previously described [24]. Tumor down-regulation was defined as less than 0.5 ratio (raw) or 0.75 (log) of the expression of normal tissue.

Genomic DNA extraction and sodium bisulfite modified sequencing

Genomic DNA samples were extracted from selected cells and tissues using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer’s instructions. Sodium bisulfite conversion of genomic DNA was done as described [31] using EZ DNA Methylation Kit (Zymo Research, Irvine, CA). Bisulfite modified sequencing was performed to determine the density of methylation in the SLC5A8 promoter. Primers for bisulfite sequencing were selected using the Methprimer software program, which identifies the location and structure of CpG islands of SLC5A8 (www.urogene.org/methprimer). A 426-base pair (bp) region of the SLC5A8 promoter from -278 to $+148$ (+1 was the translation start site) encompassing 45 CpG sites were amplified by bisulfite modified specific primers (sense:

gaggttttatattgggtttgagg, anti-sense: cccatcaaaaaatccttaaaaatcta) (Supplementary figure 1). The methylation level of each CpG site on SLC5A8 was estimated by comparing the height of the cytosine peak with the height of the thymine peak on the chromatogram as previously described [32].

Quantitative methylation specific PCR (QMSP) assay for SLC5A8

The bisulfite-modified DNA samples were used as a templates for fluorescence-based QMSP for SLC5A8, as previously described with modification [15]. In brief, primers (sense: tcgaacgtatttcgaggc, anti-sense: acaacgaatcgatttccg), and a labeled probe (6FAM-caacgacgaatacaaaaacgactaccaac-BHQ-2) were designed to specifically amplify the bisulfite-converted sequence of SLC5A8. The ratios between the expression levels of SLC5A8 and the reference gene, β -actin were used as a measure for representing the relative level of methylation of promoter DNA in the tissues (SLC5A8/ β -actin X 1000). Bisulfite-modified DNA was amplified by PCR with the following reaction conditions: 1 X buffer, 600 nM of each primer, 200 nM of probes, 0.2 mM of dNTPs, 0.5 U of Taq enzyme and 20 ng of bisulphite-modified DNA in a final volume of 20 μ l. The condition of the QMSP was as follows: 95°C for 10 min for denaturation, 55 cycles of amplification (95°C, 30 s; 60°C, 30 s; 72°C, 30 s) with a final elongation step of 10 min at 72°C. Each plate contained tissue samples and water blanks, as well as positive and negative controls. Genomic DNA extracted from a human prostate cancer cell line (DU145) cells with bisulfite treatment served as a positive control for QMSP. Serial dilutions of this DNA were used for constructing the calibration curves on each plate as previously described [22]. The more than 3 fold ratio of methylation between tumor and normal tissue was considered as hypermethylated.

Treatment of DNA methyltransferase inhibitor, 5-Aza and HDAC inhibitor, trichostatin A (TSA)

Lung cancer cell lines were treated with either 5-Aza or TSA (Sigma, St. Louis, MO), which are commonly used to determine whether DNA methylation or histone modification influence gene expressions. Briefly, cells were split to low density (1×10^6 cells/T-75 flask) 24 hours before treatments. Stock solutions of 5-Aza and TSA were dissolved in phosphate-buffered saline and 100% ethanol, respectively. Cells were treated with 2 mM 5-Aza for 5 days or 300 nM TSA for 24 hours. We also mock treated cells with the same volume of phosphate-buffered saline or ethanol as negative controls. The medium was changed at 24 hours after addition of 5-Aza. RNA was extracted after the treatment for RT-PCR analysis. The cDNA samples were used for quantification of SLC5A8 expression using the above described primers. β -actin was co-amplified for normalization of the SLC5A8 expression.

Statistical methods

Student's t-test was used to compare levels of expression or methylation between tumor and adjacent non-tumor tissues. Reported p values are two sided. P value < 0.05 was considered statistically significant. SAS version 9 software (SAS institute, Cary, NC) was used for all statistical analyses in this study.

RESULTS

RLGS analysis in lung cancer patient samples

Differential methylation profiles were obtained from the DNA samples of lung tumor and adjacent non-tumor tissues with RLGS analyses from 26 lung cancer patients. For each patient, the non-tumor tissue profile was compared to the tumor profile. Differential methylation was detected either by the absence or decrease in signal intensity. Using the

surrounding single copy spots as an internal control, we could visually estimate the loss of intensity of a locus or quantify it by densitometry. We could reliably detect loss of spot intensity equal to 30–40% of methylation [28].

We found that 229 RLGS fragments showed hypermethylation (spots present at least one patient's normal tissue profile that was absent or decreased in intensity from that patient's tumor profile). Among these 229 loci, 108 different loci were found in only one tumor/normal pair out of all 26 subjects. Sixty eight loci were detected in 2 tumor/normal pairs, 31 loci in 3 pairs, 15 loci in 4 pairs, 5 loci in 5 pairs, and 2 loci were hypermethylated in 8 pairs, respectively. These RLGS fragment losses are indicative of hypermethylation of those loci. Supplementary table 1 shows the degree of hypermethylation in different histological subtypes of lung cancer. The percentage of hypermethylated RLGS fragments ranges from 0.05% to 2.34% among tumors. The frequencies of locus methylation by tumor cell type are followed: 186 of the 229 loci were methylated in adenocarcinoma, 109 in squamous cell carcinoma and 45 in large cell carcinoma (Supplementary figure 2). Among the 229 hypermethylated loci, information of 109 hypermethylated loci was available from previous studies [33–35]. Among these 109 sites, we found annotated genes for 69 loci. Information for chromosomal location, CpG island traits, and the genomic context were assessed (supplementary table 2). For the 69 annotated genes, most of the CpG islands from which the RLGS fragment arises were found in the promoter region at the 5' end of the gene and were not previously reported to be hypermethylated in lung cancer (supplementary table 2). Hypermethylation sites identified by RLGS were found at loci on chromosomes 2p, 2q, 3p, 4q, 5p, 6p, 10p, 17p, 17q, 18p, 18q, and 21q sites, where loss of heterozygosity has been frequently reported in lung cancer [36–39]. This analysis revealed that 15.3% (4 of 26) of the lung tumors show differential methylation in SLC5A8 (supplementary table 2).

Expression of SLC5A8 in lung cancer cell lines, tumor and adjacent non-tumor tissues

Semi-quantitative RT-PCR was used to characterize SLC5A8 expression in 8 paired tumor and adjacent non-tumor tissues (Fig. 1A) and in 22 lung cancer cell lines. The SLC5A8 was not expressed in most lung cancer cell lines (21/22) except H69 cell line (Supplementary figure 3). In addition, SLC5A8 expression in tumor tissues was reduced as compared with that in the adjacent non-tumor tissues. Quantitative real-time RT-PCR showed that the SLC5A8 was well expressed in adjacent non-tumor lung tissues, while 39.1% (9/23) of tumor tissues were either silenced or reduced (Fig. 1B). Among these 9 down-regulated tumors, 5 tumors (55.6%, 5/9) were hypermethylated and one sample (11.1%, 1/9) was hypomethylated. Conversely, 2 out of 6 over-expressed tumors were hypomethylated and hypermethylated, respectively. Different methylation status of each samples were indicated in Fig. 1B.

Treatments of 5-Aza and/or TSA restore SLC5A8 expression

To investigate a relationship between epigenetic changes and expression of SLC5A8, three lung cancer cell lines (H322, Calu3, and A549) were treated with a DNA methyltransferase inhibitor, 5-Aza, and/or a HDAC inhibitor, TSA. SLC5A8 mRNA expression was compared between the inhibitor-treated and untreated cell lines using semi-quantitative RT-PCR. Among three lung cancer cell lines which SLC5A8 expression was not detected, all three cell lines were reactivated by TSA, while only Calu3 was reactivated by 5-Aza treatment. These results imply that the SLC5A8 is suppressed by DNA methylation and/or histone modification (Fig. 2).

Quantitative methylation specific PCR (QMSP) assay and bisulfite sequencing of the promoter region of SLC5A8

QMSP was performed to evaluate the methylation status of SLC5A8 promoter in lung tissues (Fig. 3). Hypermethylation at the promoter region of SLC5A8 was primarily detected in lung tumor tissues as compared with that in adjacent non-tumor tissues (14/23, 60.9%). Average of log-transformed methylation levels, $\log [(SLC5A8/\beta\text{-actin}) \times 1000]$ in the tumor are significantly higher than those in the adjacent non-tumor tissues (-1.16 vs. -2.09 , $p < 0.01$). The distribution of methylation levels and pair-wise comparisons are graphically illustrated in Fig. 3.

The extent of methylation in the SLC5A8 promoter region was also assessed in lung cancer cell lines and tumor/adjacent non-tumor tissues by bisulfite modified sequencing. Representative sequencing results are presented in Fig. 4. In the SLC5A8-silenced A549 lung cancer cell line and tumor tissues, most of CpG sites were methylated while the CpG sites were unmethylated in the adjacent non-tumor tissues.

DISCUSSION

Among hypermethylated genes identified, SLC5A8 was suggested as a tumor suppressor gene. As previous studies described in other cancer sites [15, 16, 18–24, 27, 40], we observed DNA methylation in the promoter region of SLC5A8 and its association with gene silencing of SLC5A8 in lung cancer cell lines and tumor tissues. Among nine SLC5A8 down-regulated tumor tissues, five (56%) of them were hypermethylated, while two out of six high SLC5A8 expression group (33%) were hypermethylated ($p > 0.05$). In addition, the SLC5A8 expression could be restored by treatment with the demethylating agent, 5-Aza (Calu3); HDAC inhibitor, TSA (H322, Calu3 and A549) or both inhibitors. Therefore, epigenetic gene silencing of SLC5A8, which is reflected by aberrant SLC5A8 methylation and/or histone modification, could represent a principal mechanism for inactivating this gene in lung tumor.

Since the SLC5A8 was firstly proposed as a tumor suppressor gene in colon cancer, many studies have reported methylated SLC5A8 in different cancer sites, such as brain, thyroid, gastric, colon, prostate, pancreas, breast and head & neck [11, 15, 16, 18–24] based upon QMSP which detect hypermethylated CpG sites responsible for SLC5A8 down-regulation. However, data regarding the relationship between DNA methylation and expression of SLC5A8 in lung tumor were not reported yet. Therefore, our present findings demonstrate the first evidence for epigenetic regulation of SLC5A8 in lung cancer, providing new insights into the role of SLC5A8 in tumorigenesis.

Li *et al.* [15] reported that 59% of colon tumors were methylated while only 5% of normal tissues were methylated. Moreover, DNA methylation in SLC5A8 appears an early event in carcinogenesis. Two independent studies reported that 60~80% of precursor lesions were methylated in SLC5A8 [4, 15]. Expression of SLC5A8 is also associated with survival of colon cancer patients. Paroder *et al.* [21] reported that expression of SLC5A8 in colon samples from colorectal cancer patients correlates with survival.

In thyroid cancer studies, 26–63% of thyroid tumor tissues were methylated [19, 20, 41]. Thyroid cell lines, which do not express SLC5A8, were densely methylated and reactivated by 5-Aza [20]. However, DNA methylation in SLC5A8 may not be an early event in thyroid tumorigenesis. Schagdarsurengin *et al.* [41] observed no methylation in the follicular adenoma (0/10). Hu *et al.* [20] reported that aberrant methylation of SLC5A8 is associated with progression of thyroid cancer although these results were not confirmed by other investigators [41].

Ueno *et al.* [16] reported that 30% (23/71) of gastric tumor tissues and most gastric cell lines (10/12) are methylated. Among the methylated cell lines, SLC5A8 was not expressed in most of the methylated cell lines. Methylated cell lines, which did not express SLC5A8, were reactivated by 5-Aza. Similar results were reported in prostate and pancreatic tumor, thus demonstrating that frequent methylation and low expression of the SLC5A8 gene product is observed in tumor tissues across cancer sites [22, 24] (Table 2).

The tumor suppressive role of SLC5A8 was suggested by Ganapathy *et al.* [17]. The SLC5A8 is a plasma membrane transporter belonging to the Na⁺-coupled transporter gene family. The transporter mediates Na⁺-dependent and electrophilic transport of monocarboxylates such as, short-chain fatty acids. Butyrate, one of the substrates for SLC5A8, is a histone deacetylase inhibitor and is known to induce apoptosis in a variety of tumors. SLC5A8 mediates the concentrative entry of butyrate into epithelial cells. Consequently, the transport function of SLC5A8 has the ability to influence the acetylation status of histones and hence gene expression in cells. The ability of SLC5A8 to deliver butyrate into epithelial cells most likely underlies the tumor suppressive role of this transporter [9].

As we demonstrated, expression of SLC5A8 was reactivated by 5-Aza and/or TSA, which are used in clinical trials for various human cancers. Rationale for this approach is that this agent reactivates the defective expression of tumor suppressor genes. Previous studies reported that over-expression of SLC5A8 induced apoptosis in breast [11], and colon cancer cell lines [42]. Our data support that such a strategy might be a potential therapy for lung cancer.

In conclusion, the present study suggests that SLC5A8 plays an important role as a tumor suppressor in lung tumor and the reduced expression of SLC5A8 by promoter methylation could become a major molecular target for the diagnosis and treatment of lung cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5-Aza	5-azacytidine
HDAC	Histone deacetylase
QMSP	Quantitative methylation specific PCR
RLGS	Restriction landmark genomic scanning
RT-PCR	Reverse transcription-polymerase chain reaction
iodide transporter	Solute carrier family 5
SLC5A8	member 8

TSA

Trichostatin A

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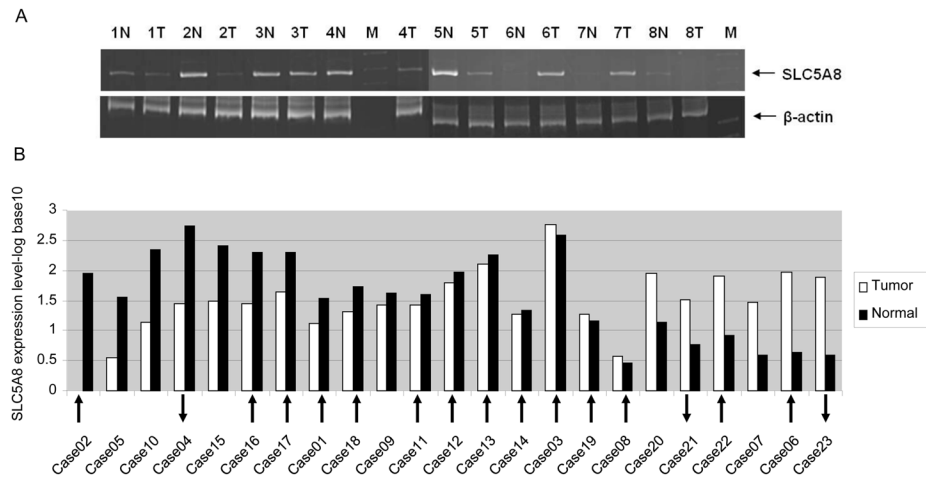


Fig. 1. SLC5A8 expression from RT-PCR analysis. (A) Representative semi-quantitative RT-PCR results of SLC5A8 transcripts from RNA isolated from 8 pairs of tumor (T) and adjacent non-tumor (N) tissues. M: DNA marker, Lanes 1~16: RNA from 8 tumor (T) and adjacent non-tumor tissue (N) pairs. (B) Quantitative real-time RT-PCR results of SLC5A8 transcripts from RNA isolated from 23 pairs of tumor and adjacent non-tumor tissues. 9 out of 23 tumor tissues were significantly down-regulated. Arrows indicate the status of methylation of tumor as compared with adjacent non-tumor tissues. Direction of arrow indicates either hypermethylation or hypomethylation.

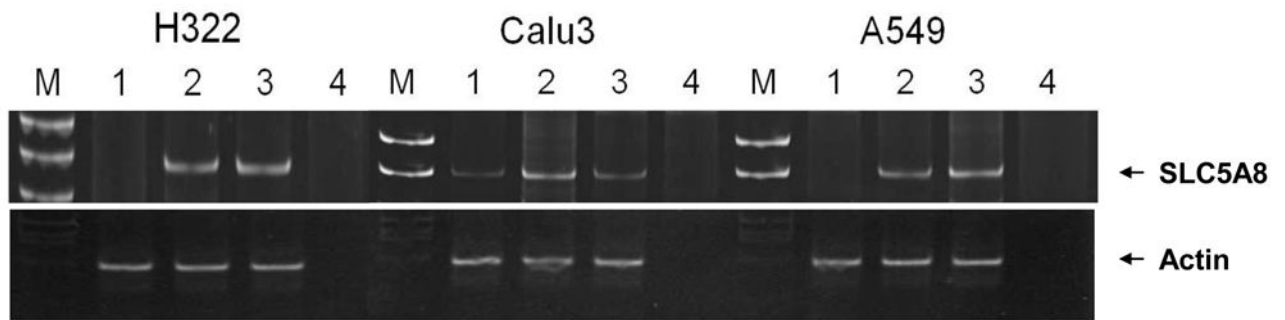
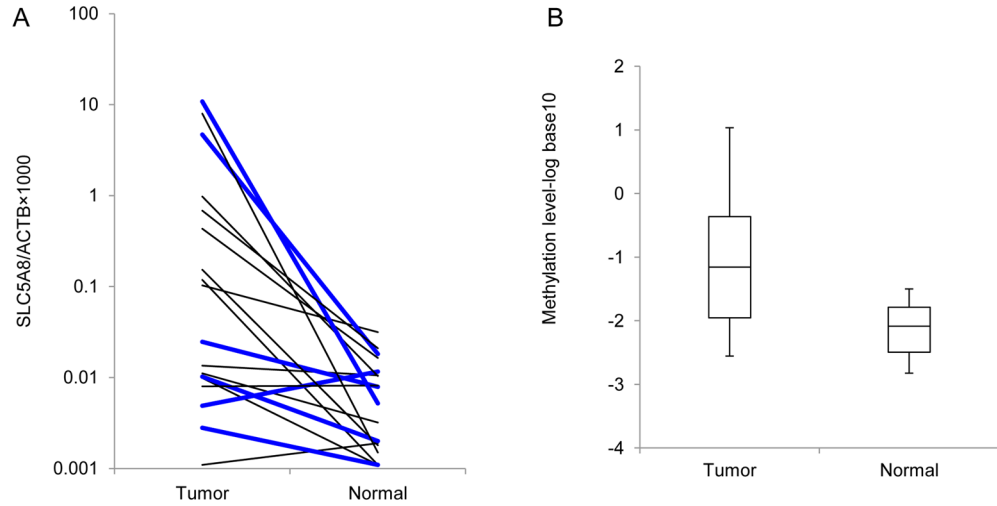


Fig. 2. RT-PCR results of SLC5A8 transcripts from RNA isolated from lung cancer cell lines with either 5-Aza or TSA treatment. Lane 1: 5-Aza treatment, Lane 2: TSA treatment, Lane 3: 5-Aza and TSA treatment, Lane 4: negative control.

**Fig. 3.**

Results of QMSP analysis. (A) Methylation levels of SLC5A8 in tumor and adjacent non-tumor tissues from lung cancer patients were measured by QMSP. Calculation of SLC5A8/ β -actin ratios was based on the fluorescence emission intensity values for both SLC5A8 and β -actin obtained by quantitative real-time PCR analysis. The relative amount of methylated promoter DNA was higher in tumor compared with adjacent non-tumor tissues (14/23, 60.9%). Results of 5 pairs were excluded because methylation levels of one of these pairs were below the limit of detection. Bold lines represent low SLC5A8 expression in tumors. (B) Distribution of methylation level in two groups is presented. Average methylation level (log10) is -1.16 and -2.09 in tumor and adjacent non-tumor tissues, respectively.

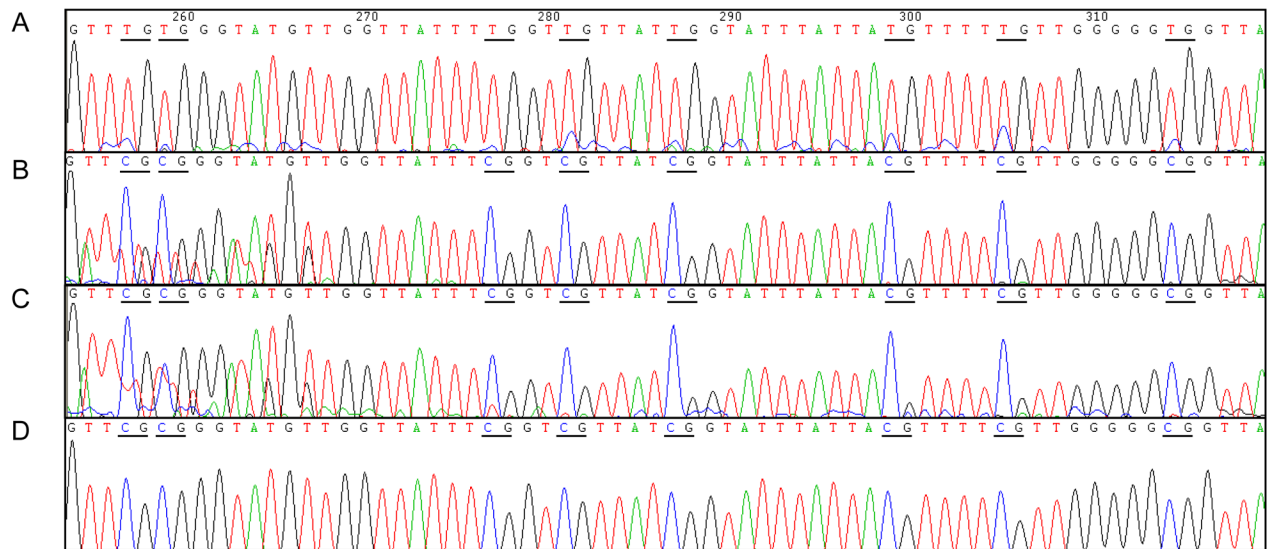


Fig. 4. Representative sequencing results demonstrate methylation status in the promoter region of SLC5A8 in DNA from adjacent non-tumor tissue, tumor tissue, lung cancer cell line, and DU145 cell. (A) Bisulfite-modified DNA sequence of SLC5A8 expressing adjacent non-tumor tissue. (B) Methylation of CpG sites in tumor tissue (C) Methylation of CpG sites in A549 lung cancer cell line. (D) Methylation of DU145 prostate cancer cell line was assessed as a positive control.

Table 1

Patient	Age	Sex	Race	Stage	Tumor type ¹	Methylated ²
1	82	M	Caucasian	III	AD	38
2	83	M	Caucasian	II	AD	1
3	87	F	Caucasian	I	AD	29
4	87	M	Caucasian	I	AD	47
5	74	M	Caucasian	I	AD	14
6 ³	74	M	Caucasian	I	AD	9
7	55	F	Caucasian	III	AD	29
8	63	M	Caucasian	II	AD	23
9	51	M	Caucasian	II	AD	7
10	73	F	Caucasian	I	AD	13
11	76	M	Caucasian	I	AD	5
12	83	M	Caucasian	I	AD	7
13	79	F	Caucasian	I	AD	25
14 ³	60	F	African American	I	AD	7
15	64	M	Caucasian	I	SCC	19
16	85	F	Caucasian	I	SCC	14
17	69	F	Caucasian	II	SCC	9
18	80	M	Caucasian	I	SCC	5
19 ³	64	M	Caucasian	III	SCC	6
20	66	M	Caucasian	I	SCC	40
21	78	F	Caucasian	I	SCC	16
22	72	M	Caucasian	I	SCC	21
23	78	M	Caucasian	II	SCC	5
24	70	M	Caucasian	I	LC	38
25	74	M	Caucasian	III	LC	5
26	58	M	Caucasian	III	LC	6

¹ AD: Adenocarcinoma, SCC: Squamous cell carcinoma, LC: Large cell carcinoma

² Number of hypermethylated loci identified by RLGS

³Samples were excluded from QMSP

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Table 2
DNA methylation in the promoter region of SLC5A8 and expression in various cancer sites

Samples	Methylation	Method	Low expression	Method	Reactivation by 5-Aza
Brain cell lines	2/2 (100) ¹	Sequencing ²	2/2 (100)	RT-PCR	2/2 (100)
Brain tumor	28/40 (70)	RLGS	13/13 (100)	RT-PCR	
Breast tumor			27/30 (90)	Tissue array	
Breast cell lines (transformed)			3/3 (100)	RT-PCR	3/3 (100)
Breast cell lines (non-transformed)			0/3 (0)	RT-PCR	
Colon cell lines	16/31 (52)	MSP	23/31 (74)	RT-PCR	6/8 (75)
Colon tumor	33/40 (83)	MSP			
Colon tumor	38/64 (59)	MSP			
Colon normal	3/64 (5)	MSP			
Colon adenoma	17/29 (59)	MSP			
Gastric cell lines	10/12 (83)	COBRA ³	6/7 (86)	RT-PCR	3/7 (43)
Gastric tumor	23/71 (30)	COBRA			
Head and Neck	9/40 (23)	MassARRAY			4/10 (40)
Lung tumor	1/5 (20)	RLGS			
Lung tumor	14/23 (61)	QMSP	9/23 (39)	RT-PCR	1/3 (33)
Pancreas cell lines	3/3 (100)	Sequencing	3/3 (100)	RT-PCR	3/3 (100)
Pancreas tumor	7/10 (70)	MSP	10/10 (100)	RT-PCR	
Pancreas normal	3/28 (11)	MSP	17/28 (61)	RT-PCR	
Prostate cell lines	2/2 (100)	Sequencing	2/2 (100)	RT-PCR	2/2 (100)
Prostate tumor	7/10 (70)	QMSP	7/10 (70)	RT-PCR	
Thyroid cell lines	3/3 (100)	QMSP	3/3 (100)	RT-PCR	2/3 (67)
Thyroid tumor	76/231 (33)	QMSP			
Thyroid tumor-cf	16/18 (89)	MSP	16/18 (89)	RT-PCR	
Thyroid cell lines	1/4 (25)	MSP			

Samples	Methylation	Method	Low expression	Method	Reactivation by 5-Aza
Thyroid tumor	9/34 (26)	MSP			

¹Numbers in bracket denote percentage

²Bisulphite modified sequencing

³COBRA: combined bisulfite restriction analysis