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Original Article

DNA promoter hypermethylation contributes to down-regulation of galactocerebrosidase gene in lung and head and neck cancers

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Abstract: Galactocerebrosidase (GALC) is a lysosomal enzyme responsible for glycosphingolipids degradation by-products of which are important for synthesis of apoptosis mediator ceramide. Reduced expression of GALC has been identified in human malignancies; however, molecular mechanisms underlying down-regulation of GALC expression in cancer remain unknown. We performed methylation and expression analysis on GALC gene in a panel of head and neck cancer (HNC) and lung cancer cell lines, attempting to understand the regulation of GALC in human cancer. QRT-PCR and western blot analysis were performed to detect the expression of GALC in HNC. Bisulfite DNA sequencing and real-time qMSP were used to detect the methylation of GALC in HNC and lung cancer cell lines. 5aza-dC treatment assay was used to analysis the functional effect of GALC methylation on GALC expression in HNC. Reduction or complete absence of GALC expression was observed in more than a half of the tested HNC cell lines (8/14). 7 out of 8 cell lines with down-regulated expression harbored heavy CpG island methylation, while all cell lines with abundant expression of the gene contained no methylation. Hypermethylation was also found in primary HNC tumor tissues and lung cancer cell lines whereas absent in normal oral mucosa tissues. Demethylating treatment demonstrated that 5aza-dC significantly restored GALC expression in cell lines with methylated promoter while showed no effect on cell lines without promoter hypermethylation. Our findings for the first time demonstrated that promoter hypermethylation contributed to down-regulation of GALC Gene, implicating epigenetic inactivation of GALC may play a role in tumorigenesis of cancer.

Keywords: Galactocerebrosidase, DNA methylation, lung cancer, head and neck cancer

Introduction

The lysosomal galactocerebrosidase (GALC), which is synthesized and glycosylated in the endoplasmic reticulum, catalyzes the hydrolysis of glycosphingolipids, including galactosylsphingosine (Psychosine), lactosylceramide (Lalcer) and galactocerebroside (Galcer). GALC plays a critical role in metabolism of glycosphingolipids, which is an essential structural component of the biological membrane of eukaryotic cells [1, 2]. Disruption in GALC function leads to aberration of sphingolipids metabolic system and is a major cause for several severe inheritable disorders. The well-documented disease caused by defect of GALC is Krabbe disease, also known as globoid-cell leukodystrophy, which is an autosomal recessive neurodegenerative disorder [3-5]. Mutations of GALC gene result in reduction of GALC enzyme activity, consequently leading to accumulation of cytotoxic psychosine and ultimately triggering demyelination in the nervous system by induction of apoptosis of myelin cells [6, 7]. So far, more than 70 mutations that are distributed throughout GALC gene had been found to be associated with phenotype of Krabbe disease [8]. It has also been reported that abrogation in GALC enzyme due to heterozygous deletion in GALC gene was involved in onset of primary open angle glaucoma which is characterized by progressive optic neuropathy and vision loss [9].

In addition to its structural role, GALC is suggested to involve in tumorigenesis due to its

critical function in hydrolysis of glycosphingolipids and further production of bioactive lipids. These bioactive lipids exert multiple cellular functions and affect the processes of malignant transformation and progression [10]. They constitute crucial components in cell membrane and play essential roles in intercellular and cell to stroma interactions which regulate many important biological functions of cells including cell adhesion, proliferation, differentiation and apoptosis [11-13]. Aberrant expression of glycosphingolipids has been observed in malignant cells [10] and was demonstrated to modulate cellular transmembrane signaling transduction by interfering with membrane-associated proteins [14]. Ceramide, a well-known apoptotic inducer, is an important downstream product from glycosphingolipid hydrolysis in GALC metabolic pathway [13, 15]. The mechanism of ceramide-inducing apoptosis has been demonstrated to be associated with mitochondria. Ceramide complexes can form a channel in mitochondrial membrane, impair the integrity of mitochondrial membrane, result in the release of pro-apoptotic substances and ultimately trigger programmed cell death [16]. It has been shown that increasing ceramide level together with up-regulation of GALC expression were observed in cancer cells treated by mitomycin C and induced apoptosis in a P53 independent manner [17]. In addition to its functions regulating the survival and apoptosis of cancer cells, down-regulation of GALC expression has been frequently observed in head and neck cancer [18], further implicating its potential roles in tumorigenesis of human cancer.

To identify novel epigenetically silenced genes in head and neck cancer (HNC), we performed a genome-wide scanning of hypermethylated genes in a panel of HNC cell lines using an approach combining gene expression microarray analysis and pharmacological unmasking of CpG island methylation [19]. From a list of genes identified through the scanning, GALC appeared as one of the top candidate genes potentially hypermethylated in the tested HNC cell lines. Given the biological importance of GALC metabolic system in cell signaling and apoptosis, we selected GALC for further analysis. In this study, we performed thorough expression and methylation analysis of GALC in human HNC and lung cancer, attempting to provide a mechanistic insight to GALC aberrant expression in cancer and further understand its role in human tumorigenesis.

Materials and methods

Cell lines and tumor tissues

An immortalized skin keratinocyte cell line Hacat, 14 HNC cell lines and 3 lung cancer cell lines were used in this study. All cell lines were cultured and maintained according to the standard protocols. Briefly, cell lines including Hatcat, PCI13, SCC25, SCC25cp, HN38, PCI51, Fudu and A549 were maintained in DMEM medium containing 10% (v/v) FBS and 100 U/ml of Penicillin. Cell lines including 011, 012, 013, 019, 022, 029, GLC82 and H8 were maintained in RPMI-1640 medium containing 10% (v/v) FBS and 100 U/ml of Penicillin/streptomycin. All cell lines were cultured in humidified incubator with 95% and 5% CO_2 at 37°C.

Genome DNA of frozen primary HNSCC tumors (n=3) and matched normal tissues (n=3) was prepared from the fresh tissue samples using a standard phenol-chloroform procedure and kept at -80°C until use. Informed consent was obtained from patients at the time of tissue sample collection and the study was approved by the Research Ethics Committee of the institute.

Qualitative real-time PCR (qRT-PCR)

Total RNA of cell lines was isolated using a Trizol reagent according to the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA). The cDNA was synthesized from 2 μg of total RNA by the First-Strand cDNA Synthesis kit (Invitrogen). cDNA and was further diluted by 5 times. qRT-PCR was performed using 3 μl of diluted cDNA in 20 μl PCR reaction containing 0.5 μM of each primer and 1× Syber-green Real-time PCR Mixture (Roche Diagnostics, Indianapolis, IN). Primer sequences were: AGACAACAGACGGCACTGAG as the forward and AACCACCACTCGTATCCTCG as the reverse. Amplification was performed for 45 cycles consisting of 95°C 20 s, 56°C 10 s and 72°C 1 min, with 10 min at 95°C for initial denaturation and 7 min at 72°C for final elongation. Amplification of GAPDH was used for normalization control.

Western blotting

Protein levels of GALC in HNC cell lines were detected by Western blot analysis using monoclonal antibody against GALC protein (Sigma-Aldrich). Actin was selected for normalization control. Protein preparations and Western blotting were carried out by standard procedures as we described previously [20].

Demethylating treatment

To test the effect of demethylating treatment on GALC gene expression, HNC cell lines (PCI13, SCC25, HN17B, PCI51, SCC25cp and O11) were treated with 10 mmol/L 5-aza-dC for 3 days with replacement of the drug and medium every 24 hours. Expression levels of GALC mRNA before and after demethylating treatment were examined using a quantitative real-time PCR method (qRT-PCR) as described above.

Bisulfite DNA sequencing

Cell line DNA was prepared and purified by standard phenol-chloroform procedures. DNA (2 μg) was subjected to bisulfite treatment as previously described [21], and bisulfite-treated DNA (2 ml) was amplified in a 25 ml PCR reaction containing 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 unit of AmpliTaq-Gold, 1× standard reaction buffer (Perkin Elmer, City, State) and 0.5 mM of each primer. Primers were designed to amplify both methylated and un-methylated GALC promoter DNA sequences. Primer sequences for bisulfite DNA sequencing for HNC cell lines were: 5'-GATTTTGGAATTTATTTTTTTTG (Forward) and 5'-ATAACCACTCAACCATTATATAAATCACAT (Reverse), and for lung cancer cell lines: 5'-ATGTGATTTATATAATGGTTGAGT (Forward) and 5'-AACTTATAAAACTAACCCCACG (Reverse). Amplification consisted of 40 cycles of 95°C 30 s, 56°C 30 s and 72°C 1 min, with 8 min at 95°C for initial denaturation and 5 min at 72°C for final extension. After verification by agarose gel electrophoresis, 1 ml PCR product for each DNA sample was subjected to Big-Dye cycle sequencing in an ABI 3779 DNA sequences, according to the manufacturer protocols (Applied Biosystems, Foster City, CA).

Real-time, quantitative MSP (qMSP)

qMSP assay was used to quantitatively detect GALC methylation in 13 HNC cell lines and the Hacat cell. Bisulfite DNA was amplified in a reaction containing 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 unit AmpliTaq-Gold, 1× standard reaction buffer (Applied Biosystems) and 0.5 μM of each primer. Primers and probes used for detecting the methylated GALC DNA sequence were: Forward: CGGCGTTAGTATTAGCGGTT; Reverse: AACTTTCGCTCGACGTTACC; Probe: 6FAM-CCGTCGCCGCCACGATAAAT-TAMRA. Amplification was performed in a Stratagene real-time thermal cycler M×3700 (Stratagene, Cedar Creek, TX) for 45 cycles consisting of 95°C 15 s, 60°C 30 s and 72°C 2 min, with 10 min at 95°C for initial denaturation. Amplification of the unmethylated beta-actin gene promoter was performed in different PCR reactions and used as a normalization control. Relative Methylation Index (RMI)was calculated from 2 ^-(cycle number of GALC-cycle number of actin), as described previously [21]. qMSP for each sample was performed in triplicate. An optimal RMI cut-off 2 was determined by comparing qMSP data with results from bisulfite DNA sequencing and MSP in a panel of HNC cell lines (Hacat, PCI13, HN38, PCI51 and O19) and a pair of HNC primary tissue samples (N2874 and T170) with known methylation status of GALC promoter sequences.

Statistical analysis

Correlation of gene expression and DNA methylation of GALC were assessed by Spearman correlation test (2-tailed), taking 0.05 as significance level and the analysis was conducted using SPSS 10.8 version.

Results

GALC gene is down-regulated or silenced in the cell lines of head and neck cancer

Transcriptional repression of GALC gene had been previously reported in larynx cancer [18]. To further determine the expression status of GALC gene in head and neck cancer, we analyzed both GALC mRNA and protein expression in a panel of HNC cell lines. Using qRT-PCR, absence or significantly reduced expression of GALC mRNA were detected in 9 out of 14 tested HNC cells as compared to an immortalized Hacat cell line which showed abundant GALC expression (**Figure 1A**). To extend our expression analysis to protein level, 4 HNC cell lines and Hacat cells with known GALC mRNA expres-

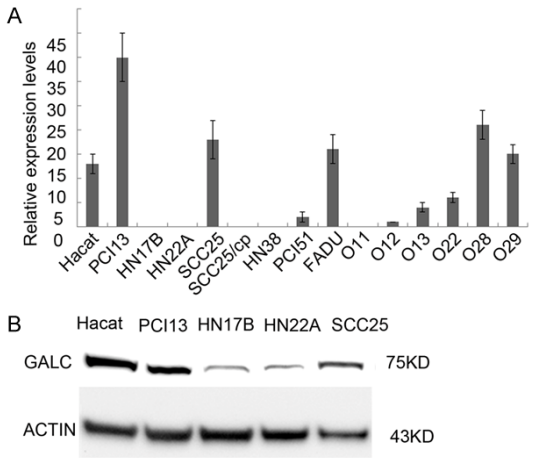

Figure 1. Down-regulation of GALC gene expression was detected in the majority of HNC cell lines. A. GALC mRNA expression was examined by qRT-PCR in 13 HNC cell lines and an immortalized skin keratinocyte cell line Hacat. GAPDH was used for normalization control; B. GALC protein expression was examined by western blotting in 4 HNC cell lines and a Hacat cell line using ACTIN as a normalization control.

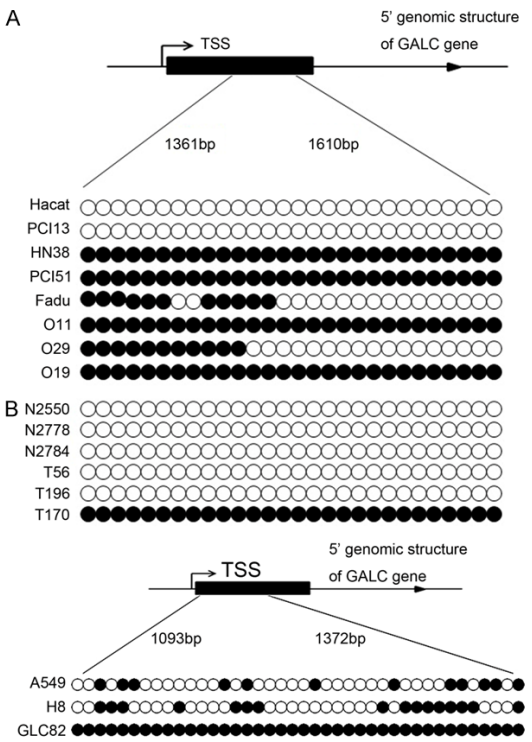

Figure 2. GALC gene promoter was hypermethylated both in head and neck cancer (HNC) and lung cancer (LC). A. bisulfite sequencing analysis was carried out to detect CpG island methylation at 5' flanking genomic region (+1361 to +1610 pb) of GALC gene promoter in cell lines with GALC expression (Hacat, PCI13, Fadu and O29), cell lines with absence of GALC expression (HN38, PCI51, O19 and O11), three primary HNC tissues and three normal oral tissues. Unmethylated CpG was indicated by unfilled circle and methylated CpG by black-filled circle; B. CpG island methylation at 5' flanking genomic region (+1093 to +1372 pb) of GALC gene promoter was examined by bisulfite sequencing in three lung cancer cell lines (A549, H8, GLC82).

sion were tested by western blot. Among 5 tested cell lines, 3 (Hacat, PCI13 and SCC25) with high levels of mRNA transcripts showed abundant protein expression while other two cell lines (HN17B and HN22A) with absence of GALC mRNA expression had significant reduction in protein levels (**Figure 1B**). This finding demonstrates that GALC protein expression is well correlated with its mRNA level, and further confirms that down-regulation of GALC expression both in protein and mRNA levels is a common event in HNC.

The promoter of GALC gene was hypermethylated both in head and neck cancer and lung cancer

To investigate if DNA promoter hypermethylation is the underlying mechanism for down-regulated expression of GALC in HNC, we performed a thorough methylation analysis on GALC gene promoter sequence. Methylation status of GALC gene promoter was first examined by bisulfite DNA sequencing in 7 HNC cell lines and Hacat cells. A total of 28 CpG islands within 5' genomic sequence around transcription starting site of GALC gene (+1361 to +1610 bp) was selected for analysis. No methylated CpG island was detected in cell lines Hacat, PCI13, HN38 and O29 that expressed high levels of GALC mRNA (**Figure 2A**). In contrast, heavily methylated CpGs were found in cell lines HN38, PCI51, O11, and O19 that had significantly reduced or absent expression of GALC transcripts (**Figure 2A**).

To verify if GALC promoter hypermethylation is also present in primary HNC tumors, we performed bisulfite DNA sequencing on 3 primary tumor samples from HNC patients and 3 normal oral tissues. GALC promoter hypermethylation was also detected in one tumor sample while absent in remaining 2 tumor and all 3 normal oral tissues (**Figure 2B**).

Although alterations of GALC have not been extensively investigated in other cancer types,

Table 1. Correlation of expression and promoter methylation of GALC gene

Cell lines	GALC expression	GALC methylation	Correlative index
Hacat	+	UM	-0.866*
PCI13	+	UM	
HN17B	-	UM	
SCC25	+	UM	
Fadu	+	UM	
O22	+	UM	
O29	+	UM	
SCC25cp	-	M	
HN38	-	M	
PCI51	-	M	
O11	-	M	
O12	-	M	
O13	-	M	
O22	-	M	

Notes: UM and M denote unmethylated and methylated GALC promoter; "+" and "-" denote meant positive and negative for GALC gene expression. Correlation analysis was conducted using Spearman correlation test (2-tailed). "*"Indicated that correlation analysis was statistically significance (*p* value <0.001).

allelic deletion of chromosome 14q31 where GALC gene is located has been frequently reported in several human cancers in addition to HNC [22-25]. To study the potential involvement of GALC promoter hypermethylation in other common cancers, we extended the methylation analysis to 3 lung cancer cell lines. Genomic sequences of +1093 bp to +1372 bp which includes 40 CpGs within GALC promoter were selected for bisulfite DNA sequencing in lung cancer cell lines. Fully methylated CpGs were detected in one cell line (GLC82, **Figure 2C**) and partially methylated CpGs were observed in remaining 2 cell lines (**Figure 2C**). This finding implicates that epigenetic alteration of GALC may not only be associated with HNC, but also contributes to tumorigenesis of other cancer types.

Promoter hypermethylation functionally down-regulated GALC gene expression in cancer

To determine if GALC promoter hypermethylation functionally regulates its gene expression, we analyzed the correlation of GALC DNA methylation and mRNA expression in 13 HNC cell lines and Hacat cells. Methylation status of the cell lines were detected by qMSP assay and mRNA expression was tested by qRT-PCR. As shown in **Table 1**, positive methylation was detected in 7 out of 14 cell lines (57%, **Table 1** and **Figure 3A**). Down-regulation or complete silencing of GALC expression was observed in all 7 cell lines with positive DNA methylation. In contrast, the majority of cell lines (6 out of 7, 86%) with absent DNA methylation displayed strongly positive GALC gene expression except for the cell line HN17B (**Table 1** and **Figure 3A**). The correlation between GALC gene methylation and expression was highly significant (correlation index was -0.866, $P<0.001$).

To further demonstrate functional relevance of epigenetic modification with GALC expression, we treated a group of HNC cell lines with demethylating agent 5-aza-dC and examined GALC expression of the treated cells using qRT-PCR. Cell lines PCI13, SCC25 and O22 with absent promoter methylation and high levels of gene expression showed no change in mRNA expression before and after 5-aza-dC treatment. In contrast, demethylating treatment dramatically restored GALC expression in cell lines SCC25cp, PCI51 and O11 with heavily methylated promoter sequence and down-regulation of gene expression (**Figure 3B**). Taken together, our findings strongly indicate that DNA promoter methylation is an essential mechanism for down-regulated expression of GALC in cancers.

Discussion

Previous studies of GALC gene have predominantly focused on its roles in neurodegenerative diseases due to its frequent germline and somatic mutations in this group of diseases. Mutations throughout the coding sequences of the gene have been found in Krabbe disease [26-28], and the majority of the mutations generates a premature stop codon leading to GALC enzyme insufficiency. Beside its demyelination effect in nervous system, emerging evidences implicate the roles of GALC in human carcinogenesis due to the frequent observation of altered glycosphingolipids metabolic pathway in human cancers [13, 29-31]. Potential association of GALC with human tumorigenesis is also supported by the findings of transcriptional repression of it expression in larynx cancer and frequent loss of chromosome 14q31 where GALC gene is located in several common cancer types [22-25]. To further determine the role

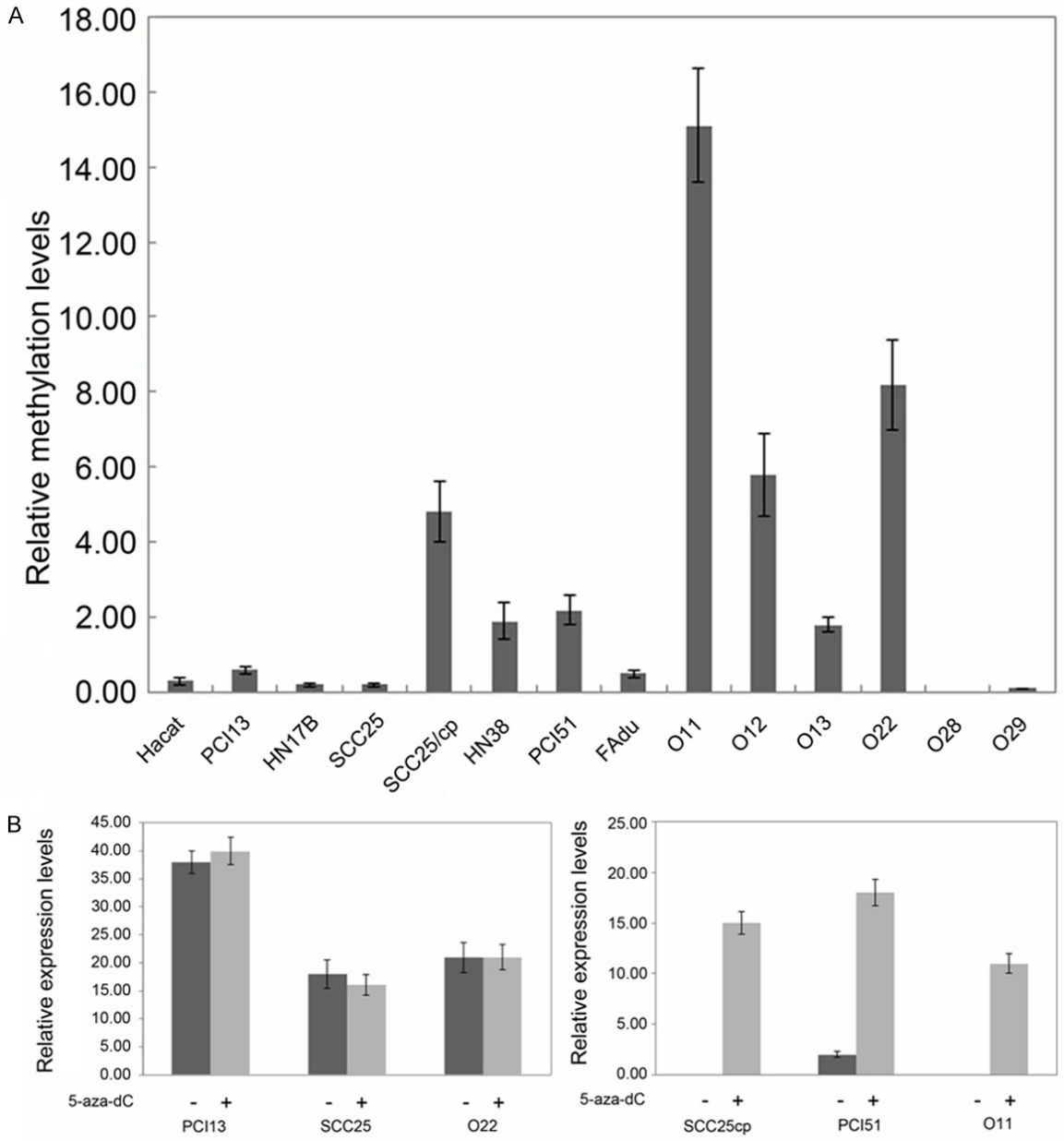

Figure 3. Promoter hypermethylation of GALC functionally correlated with its expression in HNC. A. Detection of GALC methylation in 14 HNC cell lines and Hacat cells by qMSP assay. B. Demethylating treatment by 5-aza-dC restored GALC expression in cell lines with heavily methylated GALC promoter (SCC25cp, PCI51 and O11) (*P<0.001*) and showed no effect on GALC expression in cell lines without promoter methylation (PCI13, O22 and SCC25) (*P=0.732, 0.694, 0.928*).

of GALC alteration in cancer, we analyzed GALC mRNA and protein expression in a large panel of HNC cell lines. We found that more than a half of tested cell lines (8 out of 13) had significantly reduced or completely absent GALC expression, both in mRNA and protein levels. This finding suggests that down-regulation of GALC expression is a frequent molecular event in pathogenesis of HNC.

Deciphering mechanism of GALC gene aberrant expression is essential for understanding its role in human cancer. So far, mutation of GALC gene has never been found and reported in cancer. Given the mechanistic importance of epigenetic event (DNA hypermethylation) in expression silencing of many critical cancer suppressor genes [32, 33], we hypothesized that DNA promoter hypermethylation may be

the major mechanism for down-regulation of GALC gene expression in HNC. Our hypothesis was first supported by the finding from our genome-wide hypermethylated gene scanning in a group of HNC cell lines. In this study, we performed thorough methylation and expression analysis in a relatively large panel of cell lines to determine the fundamental role of GALC gene in HNC. Both bisulfite sequencing and quantitative MSP (qMSP) detected heavy CpG island methylation in GALC promoter sequences in the majority of tested cell lines from HNC and lung cancer. Furthermore, GALC gene methylation level is inversely correlated with its mRNA expression and demethylating treatment significantly restored GALC expression in cell lines harboring methylated CpG promoter. These findings for the first time identified promoter hypermethylation as the major mechanism for down-regulation of GALC expression in HNC and implicate GALC as a putative tumor suppressor gene whose expression silencing may play a role in tumorigenesis of HNC and perhaps lung cancer as well.

In light of the critical roles of GALC associated metabolic pathway in biological functions of cells, inactivation of GALC by DNA methylation may be an important event for cancer initiation and progression. Galcer is a direct substrate hydrolyzed by GALC enzyme and ceramide serves as a key hub in GALC metabolic system. These two bioactive lipids have been demonstrated to regulate multiple functions associated with metastasis, apoptosis and drug response of cancer cells [34-36]. Therefore, GALC gene hypermethylation and functional inactivation may lead to disturbance of GALC metabolic system and subsequently initiate essential molecular events associated with malignant transformation. Thus, further studies are required to elucidate intracellular alterations of GALC metabolic system and their underlying molecular mechanisms for malignant phenotypes induced by GALC gene inactivation.

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Disclosure of conflict of interest

None.

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