



The Epigenetic Modification of *SLC5A8* in Papillary Thyroid Carcinoma and Its Effects on Clinic-Pathological Features

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Abstract

Background: Epigenetic alterations such as DNA methylation are known as the main cause of different types of cancers through inactivation of tumor suppressor genes, especially thyroid cancer. Identification of novel and effective markers are important in diagnosis and prevention of thyroid cancer. In the present study, the expression and methylation of Solute carrier family 5 member 8 (*SLC5A8*) in Papillary Thyroid Carcinoma (PTC) in comparison to multinodular goiter (MNG) have been studied.

Methods: Overall, 41 patients with PTC and 36 patients affected by MNG were recruited from four hospitals in Tehran and Qazvin, Iran in 2018. Thyroid tissues were obtained during thyroidectomy. RNA and DNA were extracted from thyroid tissues. Quantitative RT-PCR assay was performed for determining the mRNA level of *SLC5A8* while Methylation-Sensitive High-Resolution Methylation was applied for assessing the methylation status.

Results: Methylation status of three regions composed of 52 CpG islands in the promoter of *SLC5A8* gene was studied by HRM assay. *SLC5A8* level in PTC tissues was significantly downregulated in average 0.4 fold in comparison with MNG tissues ($P=0.05$). The aberrant methylation of *SLC5A8* (b) region was remarkably different in PTC and MNG cases. The promoter methylation of *SLC5A8* (c) was significantly related to *BRAF* mutations and vascular invasion in PTC patients.

Conclusion: The aberrant promoter hyper methylation of *SLC5A8* was related to aggressive PTC. Therefore, there is some evidence to support the hypothesis that *SLC5A8* could be a paly important role in the development of PTC.

Keywords: DNA methylation; Gene expression; Genes; Tumor suppressor; Thyroid cancer; Papillary



Introduction

Thyroid cancer is the fourth most common cancer worldwide. Its incidence has increased over the last decades. The percentage of all new cancer cases was 3% in 2019. These data are available at SEER databank

(<http://seer.cancer.gov/statfacts/html/thyro.html>) and this rate will increase in future years (1). Iran Cancer Data System Registry has also reported that the thyroid cancer incidence has been increased in recent years in IRAN (2). Papillary thyroid carcinoma (PTC) is one of the most important types of thyroid neoplasm which accounting for about 80% of thyroid carcinoma. Fourthly, this type of thyroid neoplasm is treatable if detected early. Therefore, identifying markers that help in the early diagnosis of this disease is very important (3, 4).

Epigenetic modifications such as methylation, histone modification, and microRNA, are contributed in the initiation and progression of cancer. Nowadays, epigenetic changes in addition to genetic changes are responsible for the initiation and progression of cancer through activation of proto-oncogenes or inactivation of tumor suppressor genes (5). Promoter hypermethylation of tumor suppressor genes and/or promoter hypomethylation of proto-oncogene plays an important role in thyroid carcinoma and become an attractive candidate in the development of cancer biomarkers (6, 7). Aberrant promoter methylation of CpG islands upstream could effect on downregulation of gene. On the other hand, DNA global hypomethylation could affect genomic stability (8, 9).

Solute carrier family 5 member 8 (*SLC5A8*) is a transporter of iodine, monocarboxylate, and short-chain fatty acids by a sodium-coupled mechanism; it is highly expressed in the thyroid gland (10). Promoter hypermethylation and gene silencing of *SLC5A8* have been frequently observed in other types of cancer such as cervical, lung, and colon cancers hence, nowadays it is a well-known tumor suppressor gene (11-14).

In the current study, we aimed to investigate whether expression and methylation of *SLC5A8* is altered in PTC tissues in comparison with multinodular goiter (MNG) tissues and found any probable correlation between this gene and clinic-pathological feature.

Materials and Methods

Sample collection

Ninety thyroid samples were resected during lobectomy and thyroidectomy from some patients who were a candidate for thyroid surgery and signed the informed consent from Yas Sepid and Atieh Hospitals (Tehran, Iran), Velayat, and Dehkhoda hospitals (Qazvin, Iran) in 2018. Samples were stored at -80 °C for subsequent analysis. Pathology reports were used to confirm the diagnosis of thyroid malignancy. Finally, 41 PTC patients and 36 MNG patients were selected. This study focused on PTC patients and MNG cases as control group, 13 non-PTC cases were excluded.

This study has been approved by the Ethics Committee of the Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Science (Ethics Code: IRI.SBMU.ENDOCRINE.RES.1397.067).

The characteristics of participants

Thirty-eight females and three males were in PTC groups. As we expected most of the thyroid cancer patients were female about 86%. Twenty-eight females and eight males were included in MNG groups. There is no significant difference in age and sex distribution between the two groups (PTCs: 41.18 ± 11.41 yr old vs. MNGs: 45.50 ± 13.27 yr old). Pathological reports showed that most of the PTCs (63.4%) were in clinical stage I. 14.6%, 9.8 %, and 2.4% PTC cases were in stage II, III, and IV, respectively. The average Tumor size was 2.18 cm. Lymph node

metastasis and vascular invasion were present in 33.3% and 43.6% PTC patients.

DNA and RNA extraction

DNA of the samples was extracted using Tissue Genomic DNA Extraction Mini Kit (Favorgen, Cat No.: FATGK001) and then was bisulfite converted with the EZ DNA Methylation-Gold Kit (Zymo Research, USA, Cat No: D5006) based on the kit's protocol.

RNX-Plus solution was used for total RNA isolation according to the kit's protocol (Cinnagen, Iran, Cat. No: RN7713C) and then was changed to cDNA using the RevertAid Reverse Transcriptase (ThermoFisher Scientific, Cat. No: MAN0012757). The quality of DNA and RNA

samples were assessed through NanoDrop-1000 (ThermoFisher Scientific, USA).

Methylation-Specific High-Resolution Methylation (MS-HRM) and Quantitative Real Time-Polymerase Change Reaction (Q RT-PCR) assays

For MS-HRM assay, three pairs and one pair specific primers were designed for studying the methylation level of *SLC5A8* promoter regions and mRNA expression level of *SLC5A8* through meth primer and primer 3 soft wares, respectively (Table 1). The MS-HRM and Quantitative RT-PCR analyses were done in the ABI Step One Plus System (Life Technologies, USA) using Real Q Plus 2x Master Mix Green (Ampliqon, Cat. No.: A325406).

Table 1: Primer sequences for MS-HRM and RT-PCR reaction

Primer sequences for MS-HRM for studying methylation					
Gene name	Locus	Forward Primer sequences	Reverse Primer sequences	T _a	CpG sites (n)
<i>SLC5A8</i>	a	GTTATATGGATAGTCG	CGCAATACTAGCC	51	10
	b	CGATGTTAGGTTATTT	CCTTAACCCAACATACC	55	32
	c	GTATTATGAATGGTGATTA	CCTCAAGCCAATAGGACCT	55	10
Primer sequences for RT-PCR					
Gene name		Forward Primer sequences	Reverse Primer sequences	T _a	
<i>SLC5A8</i>		AGCCCTGTGTATTTCGAATGG	CAGACCAACGAGTGCTCCAA	59	

Abbreviation: MS-HRM, Methylation Sensitive High Resolution Melting; T_a, appropriate annealing temperature; CpG, cytosine-phosphate diester-guanine; RT-PCR, real time polymerase chain reaction

Standard samples containing Methylated and Non-methylated DNA (Hum Diagnostics, Iran) were mixed to prepare a range of dilutions representing 75%, 50%, and 25% methylation levels in addition to 100% (Methylated) and 0% (Non-methylated) primary controls, subsequently used in each experiment to detect the methylation level of patients' samples. Standard curves of unknown samples were compared with standard curves of known samples to deduce the methylation level of each patient sample. The MS-HRM data is quantitative, so for some analysis, we need qualitative data. For this purpose, non-

methylation and methylation were defined as a methylation level less than 12.5 and methylation level more or equal to 12.5 respectively.

The efficacy of quantitative RT-PCR was studied by standard curve. Relative mRNA expressions were normalized using average of Δ Threshold Cycle (Δ CT = CT *Target gene* - CT *GAPDH*). *SLC5A8* expression in MNG samples was examined to show a normal curve. Expression of genes in each PTC sample was compared with the average expression in MNG samples. Fold change of expression was estimated using $2^{-\Delta\Delta$ CT while $\Delta\Delta$ CT stands for Δ CT of MNG cases sub-

sided by Δ CT of PTC cases. Expression data was divided into low expression (fold change less than 0.5), unchanged expression (fold change ranged from 0.5 to 2), and over expression (fold change more than 2) (15).

Statistical analysis

Normal distribution of data was evaluated by employing the Kolmogorov-Smirnov test. The variables with and without normal distribution were determined by Independent samples *t*-test and Mann Whitney U test, respectively. Correlation analyses were done using Pearson, Spearman and Chi-Square tests. Receiver Operating Characteristic Curves were utilized to show the diagnostic value of tests. The precision of area under the curve (AUC) classification for a diagnostic test was as follows; AUC between 0.9-1.0 is classified as excellent and AUCs between 0.8-0.9, 0.7-0.8, and 0.6-0.7 are classified as good, fair, and poor, respectively (16). All *P*-values were two-sided, and *P*-values less than or equal to 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism ver. 8 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS ver. 22.0 (IBM Corporation, Armonk, NY, USA).

Results

The methylation profile and mRNA expression of SLC5A8

Methylation status of three regions composed of 52 CpG islands in the promoter of *SLC5A8* gene was studied by HRM assay. When the methylation level, less than 12.5, was considered as non-methylated and methylated level more or equal to 12.5, 38 out of 41 (80.5%) PTCs and 34 out of 36 (94.4%) MNGs were not methylated in *SLC5A8* (a) region. However, the methylation level of *SLC5A8* (a) varied from 12.5% to 35% and from 12.5% to 40% in three and one methylated PTC and MNG samples, respectively. In PTC and MNG= cases, 51.2% and 58.3% were methylated in *SLC5A8* (b). Although the number of methylated cases in MNG group was higher, the highest methylation level of this region (50%) was found in PTC cases. *SLC5A8* (c) gene showed methylation in 23 out of 41 of PTCs and 14 out of 36 MNG cases. The highest methylation rate was 40% observed in one MNG case. In *SLC5A8* (a) and (c) regions, no significant difference in methylation level between PTC and MNG cases was found. The aberrant methylation of *SLC5A8* (b) in PTC cases was remarkable, although it just marginally fell out of statistical significance (*P*=0.07, Table 2).

Table 2: *SLC5A8* promoter methylation levels in PTC and MNG cases

Variable	Gene methylation						P-value
	PTC Cases			MNG Cases			
	Median \pm SE	Min-Max	Frequency	Median \pm SE	Min-Max	Frequency	
<i>SLC5A8</i> (a)	2.5 \pm 1.15	0-35	NM: 38 M: 3	0 \pm 1.44	0-40	NM: 34 M: 2	0.17
<i>SLC5A8</i> (b)	11.25 \pm 1.58	0-50	NM: 19 M: 21	12.50 \pm 1.42	0-35	NM: 15 M: 21	0.07
<i>SLC5A8</i> (c)	12.5 \pm 1.47	0-32	NM: 18 M: 23	10 \pm 1.91	0-40	NM: 22 M: 14	0.54

Abbreviation: PTC: Papillary Thyroid Carcinoma MNG: Multinodular Goiter M; Promoter methylation equal or more than 12.5, NM; Promoter methylation less than 12.5

As Fig. 1 indicates, the highest methylation level of *SLC5A8* (a) ranged from 25 to 50%, which

occurred in two PTC cases and one MNG. The methylation level of 55.6% of MNG and 43.9%

of PTC cases were zero. The methylation rate of 25% and 50% in *SLC5A8* (b) and *SLC5A8* (c) was observed mostly in MNG cases compared

with PTC cases (3 vs. 1 and 7 vs. 2 cases, respectively). The methylation level of three regions in *SLC5A8* significantly correlated with each other.

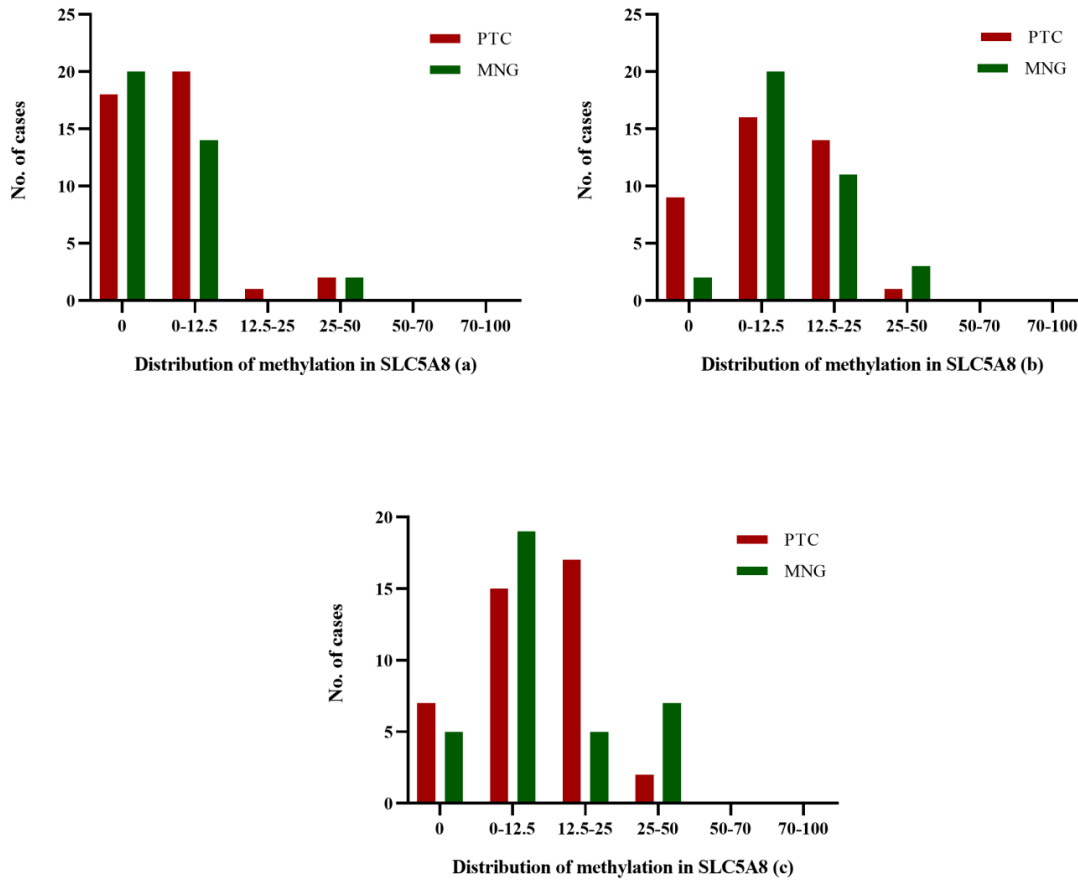


Fig. 1: Distribution of *SLC5A8* promoter methylation in different regions

The methylation status are categorized into six classes; 0:0% methylation. 0-12.5: methylation percentage <12.5%. 12.5-25: methylation percentage from ≥12.5% to <25%. 25-50: Methylation percentage from ≥25% to <50%. 50-75: Methylation percentage from ≥50% to <75%. 75-100: Methylation percentage from ≥75% to 100%. PTC: Papillary Thyroid Carcinoma MNG: Multinodular Goiter.

SLC5A8 level in PTC tissues was significantly downregulated in average 0.4 fold in comparison with MNG tissues ($P=0.050$; Fig. 2 & Table 3). Downregulation of *SLC5A8* was observed in 24 (61.5%) out of PTC cases while in 11 (28.2%) and 4 (10.3%) PTC tissues, overexpression and unchanged expression in comparison with MNG cases were observed, respectively. *SLC5A8* expression was not associated with the methylation level of any promoter region of *SLC5A8*.

Table 3: Δ CT of SLC5A8 in PTC and MNG cases

Variable	Gene expression		P-value
	Mean \pm SD	Min-Max	
PTC cases	5.36 \pm 6.78	-10.56 – 18.73	0.050*
MNG cases	2.46 \pm 5.90	-19.68 -14.40	

*Correlation is significant at the 0.05 level

PTC: Papillary Thyroid Carcinoma MNG: Multinodular Goiter

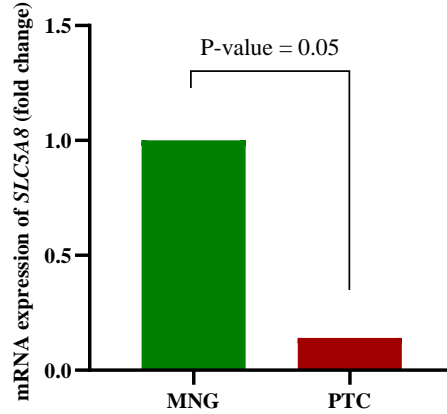


Fig. 2: Mean fold change diagram of NOL4 and SLC5A8 level in Papillary Thyroid Cancer cases in comparison with Multinodular Goiter Cases.

(a) and (b) are showing significant downregulation of NOL4 and SLC5A8 in PTC cases compared to MNG cases respectively

The relationship between methylation profile, mRNA expression of SLC5A8 and clinic-pathological features

The promoter methylation of SLC5A8 (c) was related to BRAF mutations. In other words, the promoter regions of SLC5A8(c) were significantly hyper methylated in BRAF positive patients in comparison with BRAF negative patients (16.2% vs. 8.7, P=0.02). The spearman correlation has been also confirmed these results (P=0.02). On the other hand, significant hypermethylation was detected in patients with vascular invasion in comparison with the patients without any reports about vascular invasion (17.44% vs. 11.2%, P=0.04). The spearman correlation has been also confirmed the significant correlation between hypermethylation of SLC5A8 (c) and vascular invasion (P=0.04). In a while, Methylation status of SLC5A8 (a) and SLC5A8 (b) were not related to tumor size, extra thyroid invasion, vascular invasion, and capsular invasions, lymph node me-

tastasis, clinical stage, and BRAF mutation. The SLC5A8 mRNA level did not correlate with gender, age of diagnosis, tumor size, extra thyroid invasion, vascular invasion, capsular invasion, lymph node metastasis, clinical stages, and BRAFV600E mutation, too.

SLC5A8 mRNA levels could be diagnostic markers for PTC

ROC curve analysis was used for determining the diagnostic value of biomarkers, which were significantly different between PTC and MNG cases (Fig. 3). NOL4 mRNA level showed significant differences at a cut-off of Δ CT >3.38 (sensitivity: 82.5%, specificity: 33.3%, and an AUC: 0.63) in PTC cases. (95% CI=0.5–0.7, P=0.05). SLC5A8 mRNA level showed significant differences among PTC and MNG cases with cut-off point of Δ CT >3.61 (sensitivity: 65%, specificity: 60.61%), and AUC 0.63(95% CI=0.5–0.7, P=0.04).

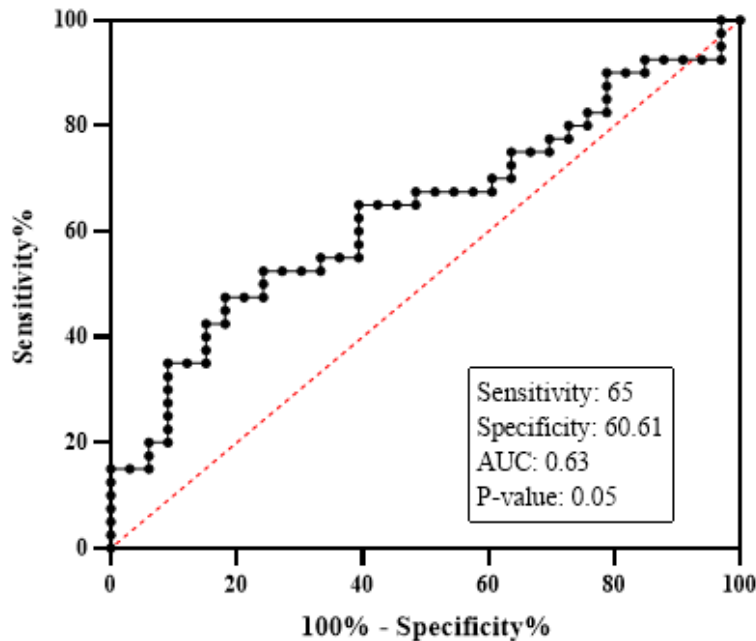


Fig. 3: Receiver operating characteristic curves (ROC) of *SLC5A8* mRNA expression biomarker (PTC vs. MNG)

The x-axis is showing 100% – specificity (false positive fraction) and the y-axis is showing sensitivity (true positive fraction). The area under an ROC curve (AUC) is used to compare the usefulness of tests. The AUC value from 0.6 to 0.7 provided a poor diagnostic value

Discussion

In the present study, in PTC and MNG samples the methylation status of promoter regions and mRNA expression of *SLC5A8* was investigated by MS-HRM. *SLC5A8* gene encodes a member of sodium-coupled symporter families transporting glucose, galactose, biotin, short-chain fatty acids, and iodine through the membrane. This transporter can transfer iodine from blood into thyroid follicular cells, so it plays a critical role in glandular function of thyroid. *SLC5A8* is notably expressed in thyroid normal tissue (10, 13, 17-19). In 2003, the function of this gene as a tumor suppressor gene was proposed in colon cancer cells (20). Thereafter, *SLC5A8* silencing was investigated in other types of cancers such as thy-

roid cancer confirming the tumor suppressor role of this protein, which is performed by inducing apoptosis (21, 22).

Hu et al. reported hypermethylation and down-regulation of *SLC5A8* in thyroid cancer cell lines (KAT-5, KAT-1k 10, and WRO). By using 5-aza-20-deoxycytidine (demethylating agent), any association between methylation and expression in WRO cell line was not approved through this correlation was supported in other cell lines (23). In our study, *SLC5A8* hypermethylation was observed in low numbers of PTC patients (34 out of 86) (18). *SLC5A8* promoter was hypermethylated in circulating free DNA (cfDNA) in PTC cases compared with goiter cases as the control group (14), which seems to be contrary with our findings. A larger sample size (57 vs. 41) and collection of high-grade samples in the previous study could affect this discrepancy.

SLC5A8 is methylated in advanced stages of PTC (14) hence lower number of patients suffering from advanced stages of cancer in this study (13.5% vs. 29.5% in the previous study) could explain lower methylation rate of PTC tissues.

We assume that different clinical partners for obtaining samples and different populations in this study can justify the low methylation rate of *SLC5A8* in these samples though, in one region of *SLC5A8* promoter, methylation was notably higher than in MNG cases. These findings suggest that methylation of *SLC5A8* promoter region can be a late event in tumorigenesis of PTC and cannot be considered as a good marker for early stages of cancer. In contrast to methylation, significant downregulation of *SLC5A8* was observed in PTC samples, which may be due to hypermethylation of other parts of this gene or other mechanisms of silencing such as histone modifications, chromatin remodeling, and effect of miRNAs or transcription factors (6).

In this study, downregulation of SLC5A8 is introduced as biomarkers of PTC though the sensitivity of SLC5A8 downregulation was lower to be ideal markers. A larger sample size from patients affected with PTC in different stages should be recruited. Based on the past studies, and the importance of this gene in pathological characteristics, the methylation of the whole promoter region of SLC5A8 must be checked. In addition, studying the methylation and expression of these genes should be assessed in other types of thyroid cancer or other types of cancer too.

Conclusion

Although we could not find significant differences between methylation status of *SLC5A8* between PTCs and MNGs cases we found a strong correlation between *SLC5A8* promoter hypermethylation and the invasive PTCs. Therefore, the present study showed some evidence to support the hypothesis that *SLC5A8* could be a play important role in development of PTC.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission,

redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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