Methylation Degrees of NIS and TIMP-3 Promoters in Well-Differentiated Thyroid Tumors

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Research

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Abstract

Background

The aim of this study was to investigate the DNA methylation pattern in CpG islands of NIS and TIMP-3 promoters in thyroid cancers and the matched non-tumoral tissues as well as multinodular goiter (MNG) tissues. This cross-sectional study was performed on total 64 patients including 28 papillary thyroid cancer (PTC), 9 follicular thyroid cancer (FTC) and 27 MNG cases. **Method**: The bisulfite sequencing PCR technique was used to evaluate the promoter methylation pattern of the NIS and TIMP-3 genes.

Results

NIS mRNA levels were decreased in both of PTC and FTC tumoral tissues compared to their adjacent normal tissues ($P = 0.04$ and $P = 0.03$, respectively). TIMP-3 expression was also reduced in both PTC and FTC tumoral tissues compared to the adjacent non-tumoral tissues ($P = 0.02$ and $P = 0.03$, respectively). Moreover, a significant reduction in TIMP-3 expression was observed in FTC tissues compared to the MNG samples ($P = 0.05$). The methylation on NIS promoter was not a common event in PTC samples, but it was frequent in FTC ($P < 0.05$). A significant hyper-methylation was shown in TIMP-3 promoter in both PTC and FTC tissues compared to the non-tumoral and MNG samples ($P < 0.05$).

Conclusion

Aberrant promoter DNA methylation suggests potential utility to differentiate benign and malignant PTC and FTC tissues for early diagnosis, and contribution for personalized clinical management and surveillance. According to the reversibility of DNA methylation, these events may be potential targets for demethylating treatments.

Background

Thyroid cancers (TCs) are the most common malignancies of endocrine system and comprise several subtypes with remarkably different biological characteristics. Among these subtypes, papillary and follicular thyroid cancers (PTC and FTC) are referred to well-differentiated thyroid cancers (WDTCs) accounting for more than 90% of all thyroid malignancies (1–3). WDTCs typically are well managed and represent favorable prognosis, however, sometimes, patients progress toward poorly-differentiated and more aggressive subtypes (4).

Nowadays, the molecular pathogenesis of TCs is not completely clear. However, environmental factors alongside the genetic alterations have been shown to play a putative role in the development of thyroid cancers (5, 6). The effect of environmental factors exerts on the regulation of gene expression which are known as epigenetic alterations (5). These alterations are specific to each histological subtype of TCs

and define their gene expression patterns and phenotypic characteristics. Therefore, understanding the details of epigenetic alterations in TCs can provide effective therapeutic strategies.

Aberrant DNA methylation has been introduced as a major epigenetic event in tumorigenesis (7, 8). Gene inactivation through hypermethylation of CpG islands located in promoter regions were described in many types of cancers (9, 10). Promoter methylation of tumor suppressor genes which are thyroid specific have been widely studied in WDTCs, however, to date, the majority of these reports are relied on the non-quantitative analysis of methylation specific PCR (MSP) methodology (9, 11–14).

Solute carrier family 5 member 5 (NIS also called SLC5A5) gene is located on chromosome 19 (19p13.11) and encodes an 80–90 kDa transmembrane glycoprotein that actively transports iodide from the bloodstream into the thyroid follicular cells (15). Thyroid-stimulating hormone (TSH) regulates NIS protein activity which consequently participates in the regulation of iodine uptake by thyroid follicular cells. In this process, a Na+/K+-ATPase pump generates a sodium gradient, transporting two Na^+ to an I^- as the first step in the thyroid hormones biosynthesis (16).

Tissue inhibitor of metalloproteinase-3 (TIMP-3), as a member of tissue inhibitors of matrix metalloproteinases (MMPs), is involved in different cellular processes like cell growth, angiogenesis, invasion, and metastasis in human cancers. TIMP-3 plays an important role in the regulation of vascular endothelial growth factor (VEGF)-mediated angiogenesis by direct binding to VEGF receptor-2 (VEGFR-2). This process prevents downstream essential signaling pathways for cell stimulation. Therefore, according to the potential angiogenesis inhibitory role of TIMP-3, it has drawn particular attention for therapeutic purposes. It has been demonstrated that hypermethylation of TIMP-3 gene was associated with clinicopathological characteristics of PTC, including extrathyroid invasion, lymph node metastasis, and advanced disease stages (III and IV) (17). TIMP-3 blocks the binding of VEGF to the receptor, and inhibit downstream signaling pathways and angiogenesis (18). Beside this, the promoter hyper-methylation of TIMP-3 gene and subsequently its down-regulation, have been observed in thyroid cancers and they are in association with aggressive pathological features (11, 19).

According to the pivotal role of NIS in the process of WDTCs pathogenesis and also in the effectiveness of the current therapeutic strategies by radioactive iodine, the objective of this paper is an investigation on NIS promoter methylation and its expression in PTC and FTC patients. Furthermore, TIMP-3 is the other studied gene in the present study owing to its targeting can be one of the essential therapeutic strategies. In the present study, DNA methylation in the promoter of these two genes including has been assessed in PTC and FTC thyroid tissues compared to matched non-tumoral as well as other benign tissues, in Iranian population.

Results

Demographic and clinicopathological characteristics

The mean ± SD of age in PTC, FTC and MNG groups were 37.89 ± 12.17, 55 ± 18.15 and 48.67 ± 14.75 years, respectively. The mean ± SD of tumor size were 1.77 ± 1 and 3.25 ± 1.81 cm in PTC and FTC groups, respectively (Table 3).

Table 3
Demographic and clinicopathological characteristics of patients

Parameters	PTC	FTC	MNG	Total
Patients number	28	9	27	64
Gender				
Male	2 (7.1)	4 (50)	8 (29.6)	14 (22.2)
Female	26 (92.9)	4 (50)	19 (70.4)	49 (77.8)
Age (years)				
< 45	21 (75)	3 (37.5)	8 (29.6)	32 (50.8)
≥ 45	7 (25)	5 (62.5)	19 (70.4)	31 (49.2)
BRAF V600E mutation				
Positive	12 (46.2)	2 (22.2)	-	-
Negative	14 (53.8)	7 (77.8)	-	-
Tumor size (cm)				
< 2	13 (46.4)	2 (25)	-	-
≥ 2	15 (53.6)	6 (75)	-	-
Exrathyroidal extension				
Positive	4 (14.8)	3 (42.9)	-	-
Negative	23 (85.2)	4 (57.1)	-	-
Extracapsular invasion				
Positive	4 (14.8)	3 (42.9)	-	-
Negative	23 (85.2)	4 (57.1)	-	-
Lymphnode metastasis				
Positive	8 (29.6)	1 (14.3)	-	-
Negative	19 (70.4)	6 (85.7)	-	-
Blood vascular invasion				
Positive	4 (14.8)	2 (71.4)		
Negative	23 (85.2)	5 (28.6)		

PTC: papillary thyroid cancer; FTC: follicular thyroid cancer; MNG: multinodular goiter; AJCC: American Joint Committee on Cancer.

Parameters	PTC	FTC	MNG	Total
TNM stage (AJCC)				
I&II	23 (82.1)	4 (57.1)	-	-
III&IV	5 (17.9)	3 (42.9)	-	-

PTC: papillary thyroid cancer; FTC: follicular thyroid cancer; MNG: multinodular goiter; AJCC: American Joint Committee on Cancer.

Analysis of NIS mRNA expression and its promoter methylation in PTC patients

The results showed that the mean of NIS mRNA level was significantly reduced in PTC tumoral tissues compared to their matched adjacent non-tumoral tissues (0.45 [0.38–5.90] vs. 1.49 [1.51–7.78], respectively, P = 0.04, Fig. 1.a). NIS mRNA level was not significantly different between PTC patients and MNG participants (0.84 [0.73–3.19] vs. 2.00 [0.98–9.26], P = 0.76, Fig. 1.b). The findings demonstrated no significant difference between total methylation status in PTC and the matched adjacent non-tumoral tissues (31.71 ± 4.54 vs 24.25 ± 4.39, P = 0.10). We also performed the analysis in each CpG site, the diffrences were not significant in none of the CpG sites (Fig. 1.c). As well as, no significant differences were observed in total methylation status (31.69 ± 4.56 vs 30.41 ± 4.24, P = 0.61) and also in none of the CpG sites between PTC and MNG tissues (Fig. 1.d).

Analysis of NIS mRNA expression and its promoter methylation in FTC patients

The NIS expression level was significantly reduced in FTC tumoral tissues compared to the matched adjacent non-tumoral tissues (0.53 [0.18–0.67] vs. 1.11 [0.19–3.16], P = 0.03, Fig. 2.a). There was not a statistically significant difference for NIS mRNA levels between FTC patients and MNG participants (0.69 [0.3–0.81] vs. 2.42 [0.17–3.7], P = 0.07, Fig. 2.b). Total methylation degree was significantly increased in FTC compared to the matched adjacent non-tumoral tissues (50.6 ± 2.67 vs. 35.06 ± 6.83, P = 0.03, Fig. 2.c). The methylation levels in FTC tumoral and the matched adjacent non-tumoral were found 69.63 ± 3.03 vs. 43.92 ± 8.63 for 6th (-955) CpG site (P = 0.03) and 50.33 ± 5.65 vs. 27.21 ± 7.68 for 7th (-963) CpG site (P = 0.02) (Fig. 2.d). However, total methylation levels of FTC tumoral tissues was significantly higher than benign tissues (50.6 ± 2.68 vs. 30.41 ± 4.24, P = 0.005, Fig. 2.e). Significant differences were observed in the methylation levels in the 4th (-947), 6th (-955) and 7th (-963) CpG sites in the forward strand of NIS promoter between FTC tumoral and MNG tissues (76.34 ± 3.12 vs. 40.43 ± 8.42, P = 0.004, 69.63 ± 3.03 vs. 23.29 ± 6.84, P = 0.001 and 50.33 ± 5.65 vs. 24 ± 6.89, P = 0.03, respectivly, Fig. 2.f).

Analysis of TIMP-3 mRNA expression and its promoter methylation in PTC patients

The mRNA levels of TIMP-3 in PTC tumoral tissues was significantly reduced compared to the matched adjacent non-tumoral tissues (0.54 [0.5–1.95] vs. 1.07 [0.96–1.79], P = 0.02, Fig. 3.a). The expression of TIMP-3 was not statistically different between PTC patients and MNG group (0.9 [0.57–1.83] vs. 0.66 [0.59–3.18], P = 0.13, Fig. 3.b). We could not found a significant difference for total methylation level of TIMP-3 promoter between PTC tumoral and the matched adjacent non-tumoral tissues (22.17 ± 1.23 vs. 18.29 ± 1.74 respectively, P = 0.07). However, analyzing ot 15 CpGs sites of TIMP-3 promoter showed that the methylation degree in the 5th (-3620) and 6th (-3625) CpGs of the forward strand of TIMP-3 promoter was significantly increased in PTC samples compared to the matched adjacent non-tumoral tissues. The methylation level in tumoral and matched adjacent non-tumoral were found 38 ± 5.71 vs.18.40 ± 1.99 (P = 0.03) for 5th site and 37.20 ± 6.02 vs.19.20 ± 4.11 (P = 0.02) for 6th site (Fig. 3.c). Total methylation degree was also in the similar level in PTC tumoral and MNG tissues (22.17 ± 1.23 vs. 22.71 ± 1.20, respectively, P = 0.84). We also observed a significant increase in the methylation degree of the 5th (-3620) CpG site in the forward strand of TIMP-3 promoter for PTC compared to the MNG tissues (38 ± 5.71 vs. 18 ± 3.97, P = 0.001, Fig. 3.d).

Analysis of TIMP-3 mRNA expression and its promoter methylation in FTC patients

TIMP-3 mRNA level in FTC tumoral tissues was significantly reduced compared to the matched adjacent non-tumoral tissues (0.49 [0.36–1.06] vs. 0.96 [0.64–1.60], P = 0.03, Fig. 4.a). A significant reduction in mRNA levels of TIMP-3 was observed in FTC tissues compared to the MNG samples (0.47 [0.35–0.92] vs. 0.89 [0.85–2.15], P = 0.05, Fig. 4.b). Total methylation level was not significantly different between FTC tumoral tissues and matched non-adjacent tumoral tissues (28.57 ± 3.42 vs. 21.02 ± 5.03, P = 0.31). We found that the methylation level in FTC samples was significantly increased in 14th (-3686) and 15th (-3689) sites of forward strand TIMP-3 gene promoter compared to the matched adjacent non-tumoral tissues. The methylation levels for the 14th CpG site in FTC and matched adjacent non-tumoral tissues was 37.46 ± 9.28 vs. 20.59 ± 7.85 (P = 0.02) and for the 15th CpG site was 52.27 ± 5.18 vs. 35.88 ± 8.26 respectively, (P = 0.03) (Fig. 4.c). Total methylation was not statistically different between tumoral tissues compared to the MNG tissues (28.57 ± 3.42 vs. 22.71 ± 3.75, P = 0.26). We also observed significantly higher methylation level in 15th CpG site (-3689) in FTC samples compared to the MNG group (52.27 ± 5.18 vs. 22.40 ± 6.81, P = 0.01, Fig. 4.d).

Association of NIS and TIMP-3 mRNA levels with clinicopathological characteristics

The mRNA level of NIS was significantly decreased in PTC patients with lymph-node metastasis compared to those without metastasis (P = 0.026, Table 4). TIMP-3 mRNA level was significantly reduced in PTC patients with age ≥ 45 years compared to those with < 45 years (P = 0.036). TIMP-3 mRNA level was also significantly reduced in PTC patients who had positive lymph-node metastasis and exrathyroidal invasion (P = 0.035 and P = 0.044, respectively, Table 4).

Table 4
The association of demographic and clinicopathological characteristic with NIS and TIMP-3 expression

	Parameter	PTC		FTC	
		Median [25th-75th]	**P**	**Median [25th-75th]**	**P**
NIS	**Lymphnode metastasis**				
	Positive	0.34 [0.06–0.41]	0.026	-	-
	Negative	0.61 [0.19–4.04]		-	
TIMP-3	**Age (years)**				
	< 45	0.06 [0.0-0.15]	0.036	1.33 [0.54–1.39]	0.095
	≥ 45	0.19 [0.08–0.42]		0.58 [0.53–0.63]	
	Exrathyroidal extension				
	Positive	0.06 [0.0-1.28]	0.035	0.65 [0.58–0.78]	0.22
	Negative	1.11 [0.50-2]		1 [0.71–1.45]	
	Extracapsular invasion				
	Positive	0.3 [0.015–0.885]	0.044	0.77 [0.66–0.91]	0.62
	Negative	1.49 [0.44–3.87]		0.87 [0.72–1.63]	

P-values are from the the Mann–Whitney U test. A P-value of < 0.05 was considered statistically significant

Discussion

It is well accepted that increased methylation may induce dedifferentiation and decrease expression of thyroid tissue-specific genes. NIS promoter is CpG rich and its methylation in these regions may cause to decrease the expression of NIS (20). The results of the present study did not show significant differences for methylation level of NIS promoter in PTC tissues compared to the corresponding none-tumoral tissues in none of the CpG sites, but the NIS mRNA level in tumoral tissues significantly decreased compared to the matched adjacent none-tumoral tissues. In addition, NIS promoter methylation level in PTCs had not significant difference compared to its methylation level in MNGs. NIS mRNA expression was not significantly different between these two groups. In previous studies, reduced expression of NIS mRNA has been shown in PTC tissues (21–23), and have been suggested as a consequent of increased promoter methylation (22). Subsequently, some studies were carried out to determine the methylation status of NIS promoter. However, nowadays, the association between the PTC development and hyper-methylation of NIS promoter is controversial (24). In this regards, in an old study, Caillous et al. (25) demonstrated that absent of iodide-concentrating ability in patients with PTC was associated with the NIS methylation. XiaoGuang et al. demonstrated that NIS promoter was hypermethylated in 27% of PTC

(16 of 60) samples compared to the control specimens (26). The methylation degree of NIS promoter in PTC tissues and in their matched adjacent non-tumoral tissues was 30.9% (47/152) and 6.58% (10/152) respectively (27). Galraro et al (15), showed that methylation of the NIS promoter is a common occurrence in malignant thyroid tumors (18 PTCs and 2 FTCs) and their surrounding tissues, but they did not find a quantitative correlation between methylation levels and mRNA expression in any groups and described a heterogeneous pattern of methylation along the CpG islands of this gene. In a study carried out using real-time quantitative methylation-specific PCR (qMSP), Stephen et al. (28) investigated 21 genes promoter methylation levels in 85 PTC, 90 FTC, 83 follicular adenoma and 65 normal groups. They did not find a significant differential methylation of NIS promoter in PTCs compared to the follicular adenoma and normal tissues taken from healthy thyroid patients. Smith et al. evaluated the methylation degrees of the NIS promoter in 32 PTCs and they found that it is not a statistically common event in the PTC tumors (9). In a meta-analysis which carried out by Zhao et al. (24), seven articles with totally 360 cases and 268 controls, were selected to investigate the relationship between NIS promoter methylation levels and PTC development. Finally they concluded that the promoter methylation in NIS gene is related to the PTC development and its aggressive and metastatic behavior. According to our results, promoter methylation of the NIS gene is not common event in PTC tumors, their matched non-tumor and benign tissues and it could not be a possible mechanism leading to reducing of NIS expression. Other regulatory mechanisms may be affected on the expression of this gene during neoplastic transformations.

According to our results, the methylation levels of the NIS promoter were significantly higher in FTC tissues compared to the matched adjacent non-tumoral. These findings were consistent with the hyper-methylation of 3th, 6th and 7th CpG positions of forward strand of the NIS promoter. In a contrary manner, mRNA expression was reduced significantly in tumoral tissues. As well as, we found highly significant results with over 20% differences in methylation levels between tumoral and benign (MNG) tissues. To the best of our knowledge, no study has investigated methylation status of NIS gene in FTC tumoral tissues compared to matched-tumoral and benign tissues, however; Stephen et al (28) found a significant differences in methylation levels of NIS promoter between FTC and normal thyroid groups. These findings imply that the NIS promoter hyper-methylation may result in reduction of its expression in FTC and can be a potential therapeutic target to restoration of iodine condensation.

In the present study, we also investigated the methylation status of TIMP-3 as a critical tumor suppressor gene in well differentiated thyroid cancers. The results demonstrated a significant increase in methylation degree at 5th (-3620) and 6th (-3625) CpG positions in the forward strand of TIMP-3 promoter in PTC tissues compared to their corresponding non-tumoral and MNG tissues. Subsequently, we found 50% decrease in TIMP-3 mRNA levels in PTC tumoral tissues compared to their corresponding non-tumoral tissues. In a study carried out on 231 PTCs by Hu et al. (11), increased methylation of TIMP-3 promoter gene was observed in 53% of patients and its association with high risk clinicopathlogical chracteristic of patients were also reported. In another study, Houqe et al (29) investigated the promoter methylation pattern of 11 genes in 23 PTC patiennts samples by qMSP technique. They reported a significant increase in methylation of TIMP-3 promoter in the PTC group compared to the noncancer tissues. Our results are similar to their findings. In the study performed by Stephen (28), as well as a significant hyper-

methylation NIS promoter, they also reported a significant increase in TIPM-3 promoter methylation in PTC tumors compared to the adenoma and normal tissues. Brait et al. (30) demonstrated 51% methylation of TIMP-3 promoter in PTC tissues, but they demonstrated that PTC tumor samples had overlapping frequencies of methylation for TIMP-3 with benign and normal thyroid samples (51% tumors vs. 42% benign vs. 27% normal). They concluded that this finding could limit the use of TIMP-3 as diagnostic biomarker for PTC and answer to whether hypermethylation present in the tumors are relevant to the neoplasia. However, the synchronization of hyper-methylation of the gene promoter and decrease of its mRNA levels in the present study and previous researches strengths this hypothesis that there is a relationship between TIMP-3 promoter methylation and PTC development.

Finally we found highly significant inversion proportion between TIMP-3 promoter methylation degrees and its mRNA expression in FTC tissues in comparison with non-tumoral and MNG tissues, more methylation in promoter lower expression in mRNA level. Our findings in this field are in accordance with Shephen et al (28) results. They reported a highly significant hyper-methylation in TIMP-3 promoter in FTC tissues compared to the adenoma and normal tissues. However, according to the limited number of studies and sample size in the mentioned area, it seems that more clinical researches with larger sample size and higher quality case-control studies are required to be taken in the future. To the best of our knowledge, this is the first study determining the promoter methylation status of these two functional genes in the selected region in tumorigenesis of well-differentiated thyroid cancers. The lack of significance may be due to the low sample numbers. In this study, we also enlighten methylation status of CpG sites to reveal specific citations for targeted therapy purposes with demethylated agents in future. However, time and budget constraints limited us to examine more CpG sites of these genes. Studying methylation levels of other CpG sites of these and other genes involved in pathogenesis of WDTCs and the mechanisms of epigenetics regulation will elucidate the role of epigenetic mechanisms in PTC and FTC development that lead to the improvement in designing effective therapies. This mater requires further investigations.

Materials And Methods

Tissue Samples

Patients who underwent surgical resection of thyroid tumor at Erfan and Atiyeh hospitals in Tehran, Iran, were selected from 2015–2016. Written informed consent was obtained from all of the patients. An experienced pathologist reviewed all tissue samples and confirmed the PTC, FTC and MNG subjects based on pathological evidence and clinical outcomes. The pathologist also separated the non-tumoral adjacent slices from tumoral samples. Overall, 64 patients met the eligibility criteria, consisting of 28 PTCs, 9 FTCs cases (tumoral tissues and the matched non-tumoral thyroid tissues from the same patients) and 27 benign cases (non-tumoral tissues from patients with multinodular goiter). Tissue samples were collected in RNase and DNase free tubes and immediately frozen in liquid nitrogen (-198 °C), and then stored at -80 °C for the later uses. Demographic and clinicopathological data of the patients were summarized in Table 1. This study was approved by the Ethics Committee of the Research

Institute of Endocrine Sciences of the Shahid Beheshti University of Medical Sciences (IR.SBMU.ENDOCRINE.REC.1395.216).

Table 1
The information of MSP primers

Genes	Accession number	Sequence 5´→3´	Start	Tm (°C)	Product size (bp)	No. CpG
NIS	NG_012930.1	F:GTGATTAGGGGATTATAGTGTATGG	-775	57.13	210	7
		R:TAAATTACAAATTTATTAAACTCCC	-988	52.69		
TIMP-3	NG_009117.1	F:TGGTTTGGGTTAGAGATATTTAGTG	-3544	57.84	172	15
		R:TTCAAATCCTTATAAAAAATAATACC	-3715	53.92		

Genomic DNA extraction

Genomic DNA was isolated according to the manufacturer's instructions from the fresh frozen thyroid tissue specimens using the FavorPrep Tissue Genomic DNA Extraction Mini Kit, USA. Then, DNA quality and quantity were determined using the NanoDrop 1000c (Thermo Scientific, USA) with considering the 260/280 absorbance ratio. All the extracted DNAs were stored at − 80 °C.

Design of primers

Met-primer online Software was used to design primers for hot-start PCR. These primers were designed for the promoter regions of NIS and TIMP-3 genes. In summary, 1000 nucleotides upstream of the start codon were selected from genome databases. Islands in the promoters were detected according to the specific inclusion criteria including island size > 100, GC percent > 50.0, and obs/exp > 0.6, CpG. (primers were detailed in Table 1). Finally, we found 7 and 15 CpG sites for NIS and TIPM-3 promoters, respectively and investigated methylation status in these sites. In order to evaluate the overall expression of NIS and TIMP-3, primers for qRT-PCR were designed using GeneRunner software (version 4.0) and checked in NCBI Primer Blast (Table 2).

Table 2
The information of qRT-PCR primers

Genes	Accession number	Sequence 5´→3´	Tm (°C)	Product size (bp)
NIS	NM_000453.3	F:CCCAGACCAGTACATGCCTC	59.82	86
		R:TGTAAGCACAGGCCAGGAA	58.85	
TIMP-3	NM_000362.5	F:CTGACAGGTCGCGTCTATGA	59.27	105
		R:CCGATAGTTCAGCCCCTTGC	60.82	
B-actin	NM_001101.5	F:GATCAAGATCATTGCTCCTCCT	57.65	108
		R:TACTCCTGCTTGCTGATCCA	58.43	

Bisulfite modification and quantitative methylation detection

Bisulfite conversion was performed using the EZ DNA Methylation-gold™ kit (Zymo Research, USA), according to the manufacturer's protocol. The sodium bisulfite-treated genomic DNA was amplified by hot-start PCR that was carried out with TEMPase HotStar Master Mix kit (Amplicon, UK). The PCR conditions were as followings: initial step at 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 sec, annealing at 55–60 °C for 30 sec, elongation at 72 °C for 30 sec, and the finally elongation at 72 °C for 3 min. Direct DNA sequencing was used to determine the methylation pattern. The primers and purified PCR products were transferred to Kowsar Biotech Company (KBC, Iran, Tehran), using the power read DNA sequencing service. Sequencing results were received in chromatograph, FASTA, SEQ and pdf formats. The methylation percentage at each position and for each sample was calculated by mC/(mC + C) formula for all examined CpGs, following that the methylation percentage was also calculated at each CpG site.

Extraction of RNA and synthesis of cDNA

After histological control, total RNA was extracted from fresh snap-frozen tissue samples, using the TRIzol reagent (Ambion, USA), according to the manufacturer's instructions. RNA quantity and purity were assessed using the NanoDrop 1000c (Thermo Scientific, USA). Total RNA was reverse transcribed with the cDNA synthesis kit (Bio Fact TM, USA) according to the manufacturer's protocol in a SENSOQUEST, Germany thermocycler.

qRT-PCR

In order to evaluate the expression of NIS and TIMP-3 genes, quantitative reverse transcriptase Real-Time PCR (qRT-PCR) was performed using the Rotor-Gene 6000 (Corbett Research, Sydney, Australia). qRT-PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing at gene-specific temperature 60 °C for 20 sec and elongation at 72 °C for 40

sec. All experiments were performed duplicated for each sample in a total volume of 25 µl volume using the SYBR Green master mix (Bio Fact, Korea).

Statistical analysis

Graph Pad Prism 8.0.1 was used for statistical analyses. Kolmogorov-Smirnov-Test was used to test the normal distribution of the data. Non-normally distributed variable data were analyzed with Wilcoxon rank test to assess paired comparisons and Mann–Whitney U test for unpaired comparisons. Methylation results are presented with mean and standard error measurement of mean. The mRNA expression results are expressed with Median and 95% confidence interval. For all comparisons p-values ≤ 0.05 were considered as statistically significant. Relative quantitation of mRNA levels of NIS and TIMP3 was performed by the comparative Ct method using the $2^{-\Delta\Delta CT}$ method (31).

Abbreviations

FTC
follicular thyroid cancer
MMPs
matrix metalloproteinases
MNG
multinodular goiter
MSP
methylation specific PCR
NIS
sodium iodide symporter
PTC
papillary thyroid cancer
SLC5A5
solute carrier family 5 member 5
TCs
thyroid cancers
TIMP-3
tissue inhibitor of metalloproteinase-3
TSH
thyroid-stimulating hormone
VEGF
vascular endothelial growth factor
VEGFR-2
VEGF receptor-2
WDTCs
well-differentiated thyroid cancers

Declarations

Ethics Approval and Consent to Participate

This study was executed under the supervision of an ethical committee of Cellular and Molecular Endocrine Research Center, Research Institute of Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Consent for Publication

Not applicable.

Availability of Data and Material

The data that support the findings of this study are available from the corresponding authors, MH and ZN, upon reasonable request.

Competing of Interest

The authors declare no conflict of interest, financial or otherwise.

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Authors' contributions

N.A. carried out the experiments and drafted the manuscript. M.Z. designed the experiments and helped in clinical samples collection. Z.N. drafted the manuscript, designed the study and helped to perform the experiments. S.S. helped in real-time PCR. M.S. helped to design the primers for methylation evaluation. S.A.F. investigated clinical samples and reported pathological results. M.H. created the hypothesis, supervised the project, and finally revised the manuscript.

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Figures

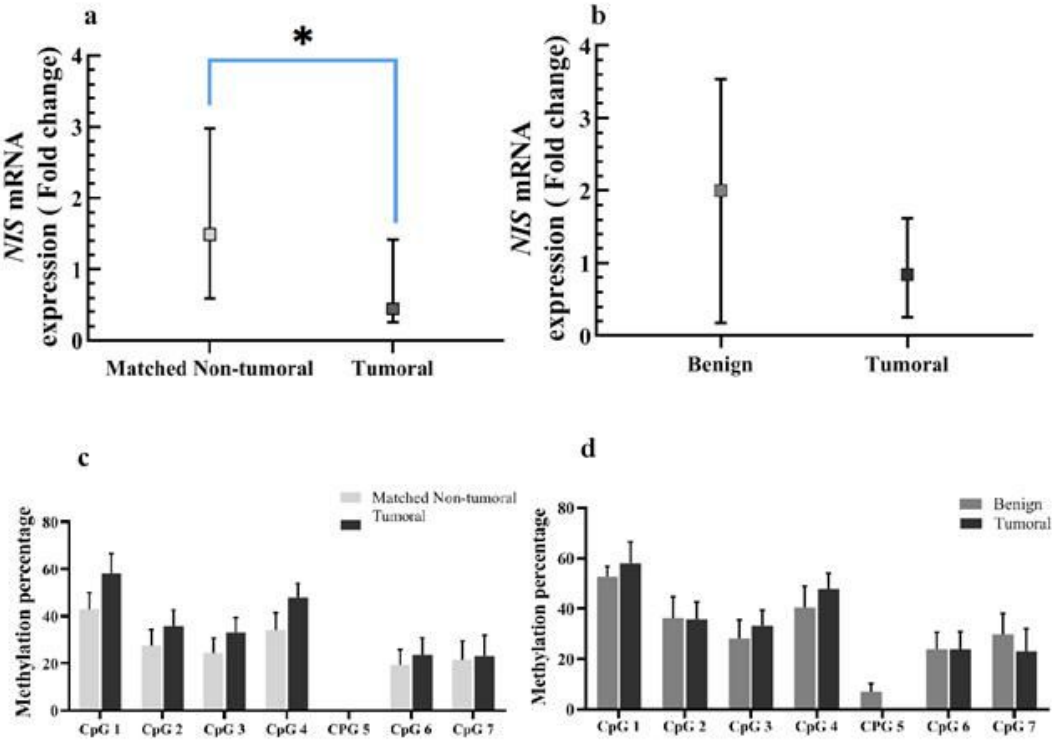

Figure 1

a. NIS mRNA levels in PTC tissues compared to its expression in the matched adjacent non-tumoral tissues (P=0.04). b. NIS mRNA levels in PTC tissues compared to its expression in the benign (MNG) tissues (P=0.76). Data are presented as Median±CI 95%. c. Total methylation of 7 CpG of NIS promoter in PTC tissues compared to the methylation degree in matched adjacent non-tumoral tissues (P=0.10). d. Methylation percentage of 7 CpGs sites of NIS promoter in PTC tumoral in comparison to the benign tissues (P=0.61). Data are presented as Mean±SEM.

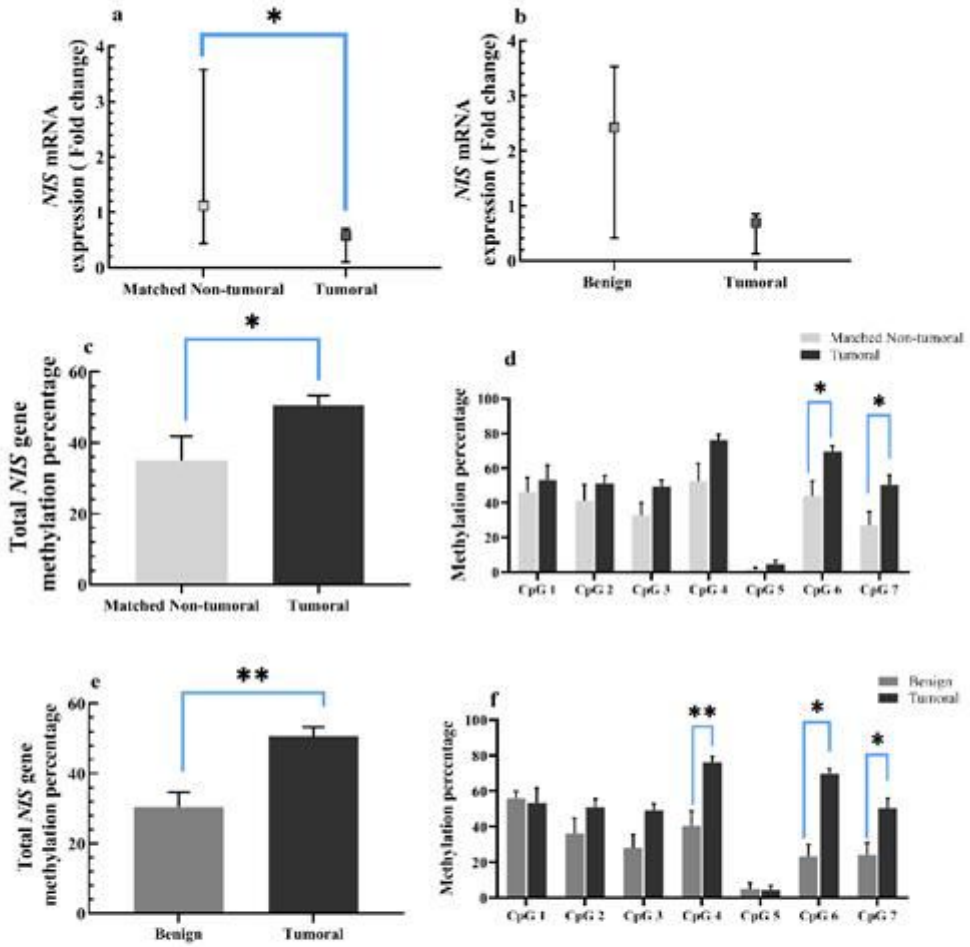

Figure 2

a. NIS mRNA levels in FTC tissues compared to its expression in the matched adjacent non-tumoral tissues (P=0.03). b. NIS mRNA levels in FTC tissues compared to its expression in the benign (MNG) tissues (P=0.07). Data are presented as Median±CI 95%. c. Total methylation of NIS promoter in FTC tissues compared to the methylation degree in matched adjacent non-tumoral tissues (P=0.03). d. The methylation levels in FTC tumoral and the matched adjacent non-tumoral of 6th (-955) CpG site (P =0.03) and of 7th (-963) CpG site (P=0.02).e. Total methylation percentage of NIS promoter in FTC tumoral in comparison to the benign tissues (P=0.001). Data are presented as Mean±SEM. f. The methylation degrees in the 4th (-947), 6th (-955) and 7th (-963) CpG sites in the forward strand of NIS promoter between FTC tumoral and MNG tissues (P=0.004, P=0.001 and P=0.03, respectivly).

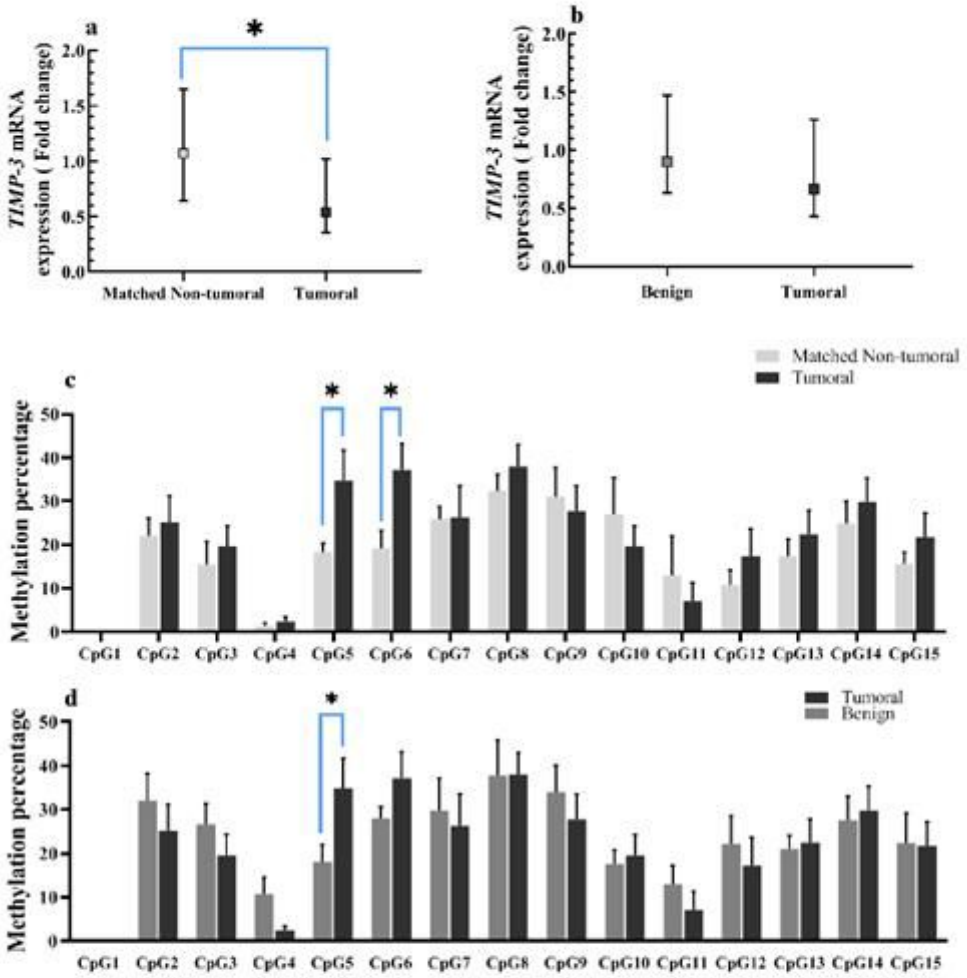

Figure 3

a. TIMP-3 mRNA levels in PTC tissues compared to its expression in the matched adjacent non-tumoral tissues (P=0.02). b. TIMP-3 mRNA levels in PTC tissues compared to its expression in the benign (MNG) tissues (P=0.13). Data are presented as Median±CI 95%. c. The methylation degree in the 5th (-3620) (P=0.03) and 6th (-3625) (P=0.02) CpGs of the forward strand of TIMP-3 promoter was significantly increased in PTC samples compared to the matched adjacent non-tumoral tissues. d. a significant increase in the methylation degree of the 5th (-3620) CpG site in the forward strand of TIMP-3 promoter for PTC compared to the MNG tissues(P=0.001).Data are presented as Mean±SEM.

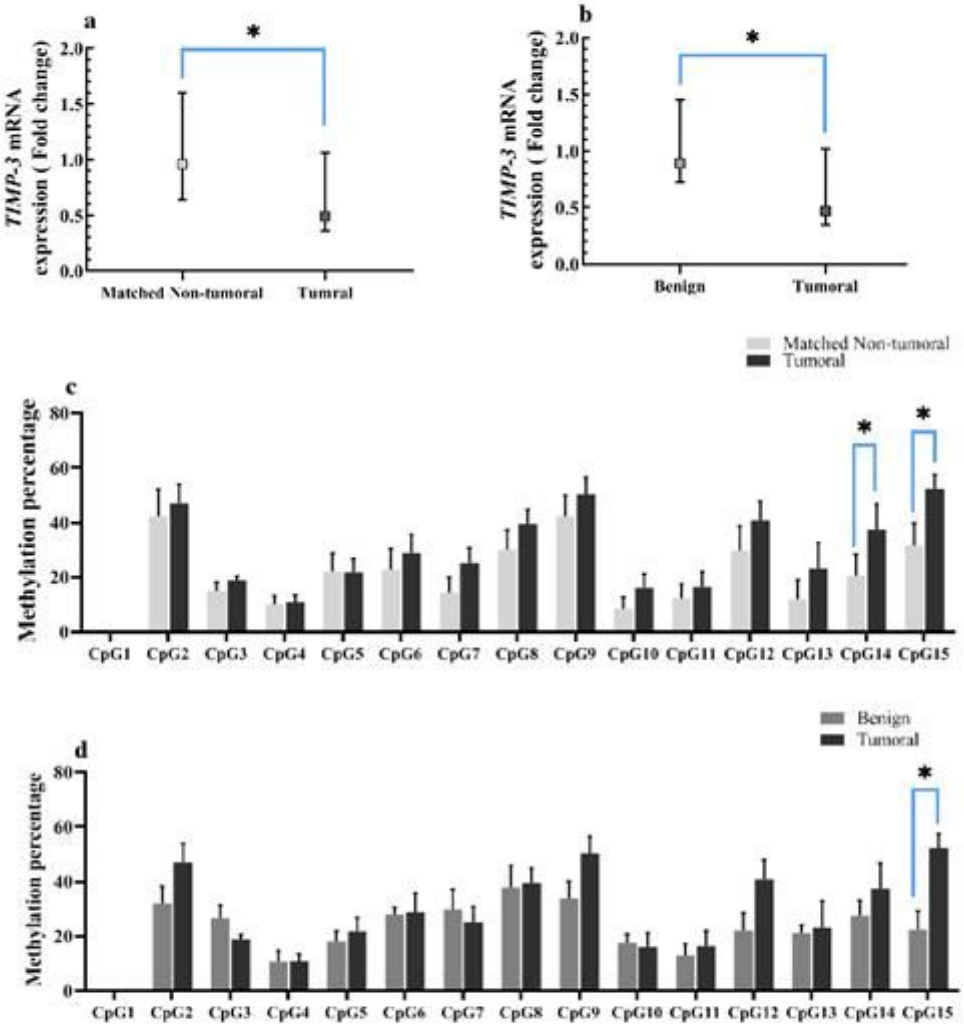

Figure 4

a. TIMP-3 mRNA levels in FTC tissues compared to its expression in the matched adjacent non-tumoral tissues (P=0.03). b. TIMP-3 mRNA levels in FTC tissues compared to its expression in the benign (MNG) tissues (P=0.05). Data are presented as Median±CI 95%. c. The methylation levels of the 14th CpG site in FTC and matched adjacent non-tumoral tissues (P=0.02) and the 15th CpG site (P=0.03). d. Significant high methylation level in 15th CpG site (-3689) in FTC samples compared to the MNG group (P=0.01). Data are presented as Mean±SEM.