

Original Article



CpG Island Methylation of the Rap1 Gap Gene in Medullary Thyroid Cancer

Bitā Faam, PhD¹; Ata. A Ghadiri, PhD^{1,2}; Mohammad Ali Ghaffari, PhD¹; Mehdi Totonchi, PhD³; Atieh Amouzegar, MD⁴; Fereidoun Azizi, MD⁴; Hajiehbi Shahbazian, MD⁵; Mahmoud Hashemitabar, PhD⁶; Seyed Ahmad Fanaei, MD⁷; Layasadat Khorsandi, PhD^{1,6*}

¹Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

²Department of Immunology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

³Department of Endocrinology and Female Infertility, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

⁴Endocrine Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁵Chronic Disease Care Research Center, School of Nursing and Midwifery, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

⁶Department of Anatomical Sciences, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

⁷Erfan Grand Hospital, Tehran, Iran

Abstract

Background: Medullary thyroid cancer (MTC) is a rare type of neuroendocrine tumor. This study aimed to investigate the gene and protein expression of *RAP1GAP* and DNA methylation patterns of its CpG74_a, CpG74_b, and CpG24 in an Iranian population with MTC.

Methods: In this case-control study, we selected 55 individuals who underwent thyroidectomy in Erfan hospital, Tehran, between 2018 and 2020. Samples were divided into normal thyroid tissues (control; n=20), benign nodule (n=20), and MTC (n=15). DNA methylation patterns were investigated using MSP (methylation-specific PCR). The protein level and mRNA expression of *RAP1GAP* were also evaluated using western blotting and real-time PCR, respectively.

Results: The hyper-methylation rates of CpG24 and CpG74_a in the MTC samples were considerably higher than the controls (83% versus 15% and 74% versus 17%, respectively; $P < 0.001$). The methylation/unmethylation ratio of CpG74_a and CpG24 was considerably higher than the controls ($P < 0.001$). The methylation/unmethylation ratio of CpG24 in the benign nodules was also considerably greater than the controls ($P < 0.001$). The mRNA expression and the protein level of *RAP1GAP* in the MTC group were considerably lower than the controls ($P = 0.005$ and $P = 0.035$, respectively). In the MTC group, aberrant methylation of CpG74_a and CpG24 was significantly correlated with decreasing expression of the *Rap1Gap* gene ($R^2: 0.23$; $P = 0.032$ and $R^2: 0.56$; $P = 0.001$, respectively).

Conclusion: Hyper-methylation in CpG24 and CpG74_a and decreasing expression of *RAP1GAP* can be considered as diagnostic biomarkers for MTC.

Keywords: CpG Island, DNA methylation, Epigenetic, Medullary thyroid cancer, *RAP1GAP*

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Introduction

Medullary thyroid cancer (MTC) [OMIM#: 155240] is a neuroendocrine tumor arising from parafollicular C cells, and it is highly resistant to radio- or chemotherapy.^{1,2} Elevated level of calcitonin is an essential feature of the MTC. Approximately 75% and 25% of MTC patients are sporadic and familial, respectively.³

Recent advances in genetic and molecular pathogenesis have led to finding molecular targets for cancer therapy or diagnosis. Cancer is a genetic alteration that disturbs the normal homeostatic stability between cell death and cell proliferation. In addition to genetic variations, epigenetic variations could be considered as key indicators of carcinogenesis.⁴ Abnormal increasing DNA methylation, as an important component of epigenetic

change, correlates with the progression of malignancies.^{5,6} Hyper-methylation suppresses the expression of tumor suppressor genes and stimulates cancer progression.^{7,8}

The *Rap1Gap* gene (OMIM#: 600278) is depleted in various human tumors such as endocrine neoplasia.⁹ The *RAP1GAP* gene encodes a type of GTPase-activating-protein (GAP) that modulates the expression of the ras-related protein 1 (RAP1). *RAP1GAP* is an important tumor suppressor gene that participates in apoptosis, cell migration, and cell-cell adhesion.¹⁰ It is down-regulated in multiple cancers such as colorectal, pancreatic, thyroid, and kidney cancers.¹¹ The role of *RAP1GAP* in human cancers is still unknown, but multiple mechanisms have been identified for the decreased level of *RAP1GAP* gene expression. DNA hyper-methylation of the *RAP1GAP*

*Corresponding Author: Layasadat Khorsandi, PhD; Department of Anatomical Sciences, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, P.O.Box: 61335, Iran. Tel: +98-613-3336380; Fax: +98-613-3336380; Email: khorsandi_cmrc@yahoo.com

gene can occur in thyroid cancer.¹² The expression of *Rap1Gap* and methylation patterns of CpG24 island and two CpG units within CpG74 (CpG74_a, and CpG74_b) in MTC tissues were investigated in the current work.

Materials and Methods

Participants

In this case-control study, 55 individuals [12 males (21.8%) and 43 females (78.2%)] were recruited. The thyroid samples were obtained from Erfan hospital, Tehran, between 2018 and 2020. The samples were selected based on outcomes of fine needle aspiration (FNA) examinations of the thyroid nodules. After confirming the diagnosis of nodules using the standard diagnostic criteria, appropriate sections of the thyroid tissues were dissected and were classified into normal, benign, or cancerous tissues. All the samples resected by thyroidectomy were evaluated histologically. The demographic characteristics of each patient were recorded in a questionnaire. The tissue samples were maintained at -80°C until use. All demographic information and thyroid hormone levels were obtained from the patients' files in the hospital archives.

Methylation-specific PCR (MSP)

The methylation status of CpG24 island and two CpG units within CpG74 (CpG74_a, and CpG74_b) of the thyroid samples were investigated in this study. It is noticeable that these CpGs are located near the transcription start site. Genomic DNA (gDNA) was extracted from the thyroid samples by a DNA Methylation-Direct kit (Zymo Research Crop, California, USA) according to the kit's instruction. In brief, 350 ng of gDNA was used in each sodium bisulfate reaction and MSP reactions were performed with a total volume of 15 µL and primers which are summarized in

Table 1. The annealing temperatures of PCR amplification were 59°C (CpG74_a), 58°C (CpG74_b) and 60°C (CpG24) for methylated pair primers and 63°C (CpG74_a), 58°C (CpG74_b) and 56°C (CpG24) for unmethylated pair primers. The reaction mixture was incubated at 95°C for 2 minutes, followed by 40 cycles of 95°C (50 seconds), annealing temperatures (55 seconds), 75°C (50 seconds), and extension at 72°C (10 minutes). Methylation-specific PCR (MSP) products were analyzed on 2.5% agarose gel.

Quantitative Gene Expression Analysis

Total RNAs were extracted using the TRIzol kit (RiboEx, GeneAll, Seoul, Korea) according to the manufacturer's guides. The isolated RNAs were converted to cDNA using a cDNA synthesis kit (Takara Bio Inc, Shiga, Japan). The generated cDNA (2 µL) was amplified in a PCR reaction mix (25 µL) containing 12.5 µL SYBR Green Master Mix (Takara Bio Inc, Shiga, Japan), and 0.2 µL of each primer (Table 2). The following program was applied to polymerase chain reaction (PCR) amplification (40 cycles): 95°C for 30 seconds, 95°C for 15 seconds, and 60°C for 20 seconds. The quantity of PCR product generated from amplification of the *RAP1GAP* gene was standardized using the quantity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) product for each sample to obtain a relative level of gene expression. Data were analyzed using the 2^{-ΔΔCT} method.

Western Blotting Analysis

The tissue samples were washed and added to radioimmunoprecipitation (RIPA) lysis buffer with protease inhibitor. The BCA assay kit (Thermo Fisher Scientific, Waltham, USA) was used to obtain protein from the cells. Lysate protein was separated on SDS-PAGE (10%) and transferred onto a PVDF membrane (Sigma, St.

Table 1. Used Primers Sequences in the qPCR Amplification and MSP Analysis

Target	Primer Sequence	AT (°C)	Product Size (bp)
GAPDH-mRNA	F: 5'-CTCATTTCCTGGTATGACAACGA-3'	61.1	121
	R: 5'-CTTCTCTTGTGCTCTTGCT-3'	58.4	
Rap1Gap-mRNA	F: 5'-ACGAGCATGTCATCAGCAAT-3'	56.4	138
	R: 5'-CCTTCTGCCAAGAAATTCA-3'	56.4	
CpG74a, MSP (U)	F: 5'-TTTTGGGTTTGGTTTTTGGTGTTT-3'	55.9	113
	R: 5'-AACACTCCACAATCATATAACATCCC-3'	60.1	
CpG74a, MSP (M)	F: 5'-TTTGGGTTTCGGTATTTGGTGTTT-3'	59.3	113
	R: 5'-ACGCTCCGCGATCATATAACGT-3'	60.3	
CpG74b, MSP (U)	F: 5'-TGAGTTTAGTTGTGTTTATTTTGG-3'	53.1	122
	R: 5'-AAAATATCCAACTCCCATCAAC-3'	55.3	
CpG74b, MSP (M)	F: 5'-CGAGTTTAGTTGCGTTTATTTTTC-3'	55.9	122
	R: 5'-ATATCCAACTCCCGTCGAC-3'	57.3	
CpG24, MSP (U)	F: 5'-TAGAGATAAAGTTTAAGAGTTGTGA-3'	54.8	220
	R: 5'-TTCTAAATCAAATAAAAACATCAA-3'	49.9	
CpG24, MSP (M)	F: 5'-TAGAGATAAAGTTTAAGAGTCGCGA-3'	58.1	220
	R: 5'-TTCTAAATCAAATAAAAACGTCGAA-3'	53.1	

M, methylated; U, un-methylated; AT, annealing temperature; bp, base pair.

Louis, MO, USA). The diluted primary antibodies (Anti-RAP1GAP, ab32119; Anti-GAPDH, ab128915; Abcam, USA) were incubated for 2 hours at room temperature. Then the cells were exposed to horseradish peroxidase-conjugated secondary antibody (ab6721, Abcam, USA) for 90 minutes at room temperature. An ECL detection kit (Sigma, St. Louis, MO, USA) was used to visualize the specific proteins. Finally, we used the ImageJ software (Version 6, Bethesda, MD) to quantitate the band density.

Statistical Analysis

The normality of distribution was checked for all variables by Kolmogorov-Smirnov analysis. Normally distributed continuous variables are reported as mean \pm SD, whereas categorical variables were summarized as frequencies and percentages. The chi-square test was applied to examine the frequencies of methylation. Quantitative variables were compared with one-way analysis of variance (ANOVA) and Tukey's post hoc test. To determine the correlation of the *RAP1GAP* gene and protein expression with DNA methylation, bivariate linear correlation analysis was done. Multinomial logistic regression was used to control the effects of potential confounders. All analyses were done using the SPSS software, version 20 (SPSS, Inc. Chicago, IL, USA). *P* values <0.05 were considered as statistically significant.

Results

Demographic and Clinical Pathologic Data of Participants

The demographic data for each group was as follows: the

control group (3 males; 17 females): 48.7 ± 10.5 years old; the benign group (6 males; 14 females): 50.5 ± 11.1 years old; and the MTC group (3 males; 12 females): 38.8 ± 12.5 years old. There was a significant difference in age distribution across these groups ($P=0.043$) (Table 2). In the MTC group, 10 cases (66.6%) had the sporadic form. The mean tumor size of MTC was 1.8 ± 0.43 cm. Metastasis was not observed in the MTC group in this study.

Methylation Patterns

The hyper-methylation pattern of selected CpGs was only seen in the sporadic form of MTC. The hyper-methylation of CpG24 in patients with MTC or benign nodules was considerably greater than the control individuals (83% versus 15% and 74% versus 15%, respectively; $P<0.001$). There was no considerable change in the hyper-methylation rate of CpG24 between the MTC and benign nodules groups. The hyper-methylation rate of the CpG74_a in MTC was considerably higher than the normal tissue (74% versus 17%, $P<0.001$) and benign nodules (74% versus 35%, $P=0.036$). The hyper-methylation rate of CpG74_b in the MTC group was not significantly higher than the controls (48% versus 36%; $P=0.062$) and benign nodules (48% versus 49%; $P=0.175$) (Table 3 and Figure 1).

The methylation/unmethylation ratio of CpG24 in patients with MTC was considerably greater than the benign nodules or normal tissue ($P<0.001$). There was a considerable elevation in the methylation/unmethylation ratio of CpG24 in the benign nodules in comparison to the controls ($P<0.001$). The methylation/unmethylation ratio of CpG74_a in MTC was considerably higher than

Table 2. Demographics and Thyroid Hormones Levels of Participants by Group

Variables	Control (n=20)	Benign (n=20)	MTC (n=15)
Age (years)	48.7 ± 10.5	50.5 ± 11.1 $P=0.121$	38.8 ± 12.5 $P=0.048^*$
Gender (females) (%)	17 (85)	14 (70) $P=0.087$	12 (80) $P=0.046^*$
BMI (kg/m ²)	21.6 ± 4.5	31.9 ± 5.7 $P=0.203$	28.1 ± 5.1 $P=0.172$
Tumor size (cm)	1.5 ± 0.24	1.7 ± 0.28 $P=0.293$	1.8 ± 0.43 $P=0.385$
T3 (ng/dL)	98.4 ± 8.7	106.6 ± 11.5 $P=0.932$	118.7 ± 10.2 $P=0.085$
T4 (μg/dL)	8.3 ± 1.9	13.7 ± 2.8 $P=0.071$	11.4 ± 2.3 $P=0.084$
TSH (IU/L)	1.7 ± 0.19	2.1 ± 0.23 $P=0.124$	2.6 ± 0.31 $P=0.217$
Calcitonin (pg/mL)	24.4 ± 3.9	33.5 ± 4.6 $P=0.064$	158.7 ± 12.3 $P=0.007^*$
SBP (mm Hg)	116 ± 11.6	124 ± 12.9 $P=0.341$	118 ± 13.4 $P=0.464$
DBP	76 ± 6.7	75 ± 7.2 $P=0.459$	78 ± 6.9 $P=0.512$

MTC, medullary thyroid cancer; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

* $P<0.05$. The quantitative variables were compared between different groups of thyroid tissues with one-way analysis of variance (ANOVA).

Table 3. Methylation Status of CpG24, CpG74_a and CpG74_b in Different Groups of Thyroid Tissues

Groups		CpG24	CpG74 _a	CpG74 _b
Control	M	3 (15%)	4 (17%)	7 (365)
	M/U	0.17	0.20	0.56
Benign	M	15 (75%), $P<0.001$	7 (35%), $P=0.053$	10 (49%), $P=0.092$
	M/U	3.00, $P<0.001$	0.53, $P=0.056$	0.96, $P=0.102$
MTC	M	12 (83%), $P<0.001$	11 (74%), $P<0.001$	7 (48%), $P=0.062$
	M/U	4.9, $P<0.001$	2.8, $P<0.001$	0.92, $P=0.431$

M, Methylated; U, Un-methylated.

Chi-square test was applied to examine the methylation status in different groups.

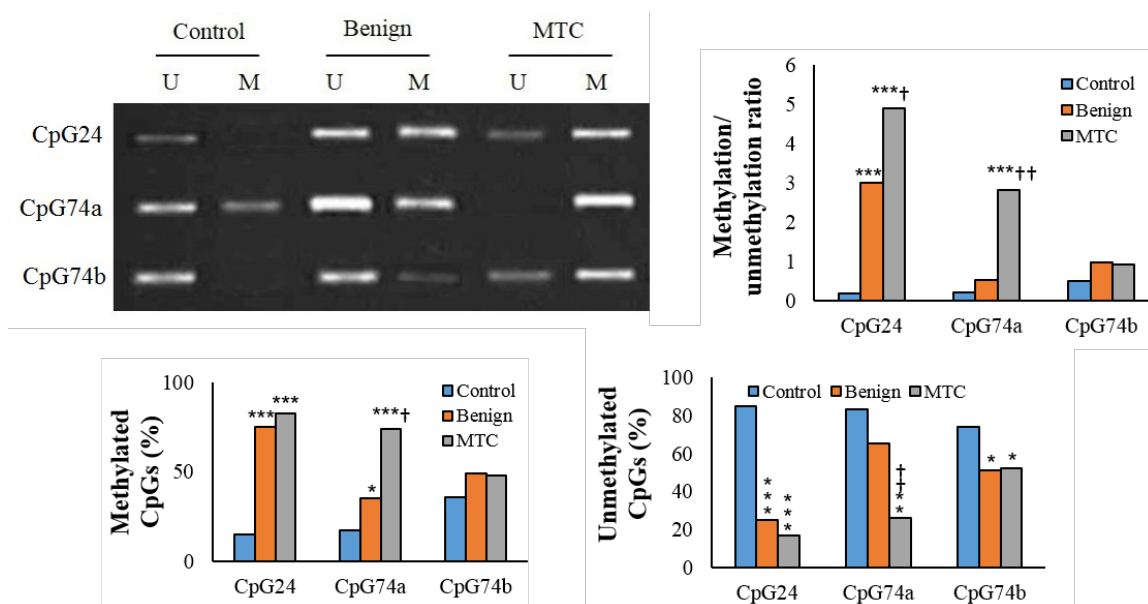


Figure 1. CpGs Methylation Pattern in Different Groups (M: Methylated; U: Un-methylated). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$. *and † indicate comparison to benign nodules and normal thyroid tissue (control), respectively.

the controls and benign nodule groups ($P < 0.001$). The methylation/unmethylation ratio of CpG74_b in MTC was not considerably greater than the normal tissue ($P = 0.431$). The methylation/unmethylation ratio of the CpG74_a and CpG74_b in benign nodules was not significantly different from the controls (Figure 1 and Table 3).

We also evaluated the methylation status of these CpGs simultaneously. Accordingly, all three CpGs were considerably hyper-methylated in patients with MTC compared to the normal thyroid tissues (31% versus 4%; $P < 0.001$). The hyper-methylation of two CpGs in the MTC group was considerably higher than the normal thyroid (69% versus 30%, $P < 0.008$) and benign nodules (69% versus 41%, $P = 0.032$). The hyper-methylation of only one CpG in MTC was noticeably lower than the normal group (9% versus 52%, $P < 0.001$). Unmethylated patterns for all three CpGs were not seen in the MTC group ($P < 0.001$). The unmethylated pattern in the benign nodules was considerably lower than the normal thyroid samples (4.8% versus 16%, $P = 0.016$). The majority of patients with MTC had a hyper-methylated pattern in two CpGs (Table 4 and Figure 2).

To assess the role of demographic and hormonal variables in the hyper-methylation status of selected CpGs, we performed five multinomial logistic regression models (Table 5).

The hyper-methylation rate of CpG24 was associated with MTC (odds ratio [OR]: 1.23; 95% confidence interval [CI]: 0.21–1.85; $P = 0.045$). This association remained significant after adjustment for sex and age (OR: 1.65; 95% CI: 0.75–2.11; $P = 0.022$). Patients with MTC had a higher risk of hyper-methylation in the CpG74_a (OR: 1.34; 95% CI: 0.23–1.52; $P = 0.046$). This association was also significant after adjustment for sex and age (OR: 1.45; 95% CI: 0.32–1.25; $P = 0.035$). There was not any association between CpG74_b hyper-methylation and MTC after

Table 4. Simultaneously Hyper-methylation Frequencies of CpGs in Different Groups

Groups	3 CpGs	2 CpGs	1 CpG	0 CpG
Control	1 (4%)	6 (30%)	11 (52%)	3 (16%)
Benign	5 (22%)	8 (41%)	7 (32%)	1 (4.8%)
	$P < 0.001$	$P = 0.052$	$P = 0.049$	$P = 0.016$
MTC	5 (31%)	10 (69%)	2 (9%)	0 (0%)
	$P < 0.001$	$P = 0.008$	$P < 0.001$	$P < 0.001$

MTC, medullary thyroid cancer.

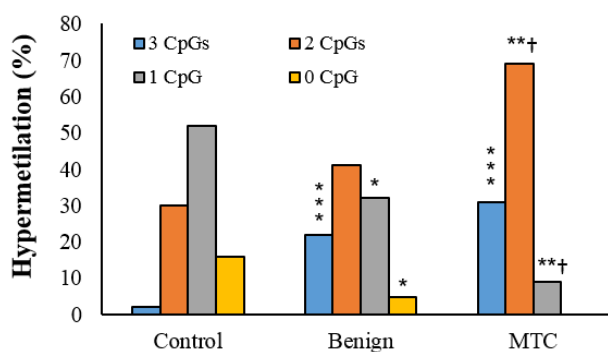


Figure 2. Simultaneous Hyper-methylation Frequencies of CpGs in Different Groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, † $P < 0.05$ and † indicate comparison to benign nodules and normal thyroid tissue (control), respectively.

adjustment for sex and age.

The association between MTC and hyper-methylation of CpG74_a was also significant after adjustment for confounding variables such as sex, age, and calcitonin level (model V) (OR: 1.92; 95% CI: 0.67–2.31; $P < 0.001$). The risk of aberrant DNA hyper-methylation of CpG74_b in the MTC group was only seen after adjustment for confounders in model V (OR: 1.58; 95% CI: 0.25–4.81; $P = 0.032$). Information about the other models is available in Table 5.

RAP1GAP Gene Expression

The expression of the *RAP1GAP* gene in MTC tissue samples was 3.2-folds lower than the controls (0.31 ± 0.08 versus 1 ± 0.00 , $P=0.005$). Benign nodules showed no noticeable change in the expression of the *RAP1GAP* gene compared to the normal thyroid tissue ($P=0.069$). The expression of *Rap1Gap* in the MTC samples was 2.9-folds lower than the benign nodules (0.31 ± 0.08 versus 0.9 ± 0.12 , $P=0.041$) (Figure 3).

In the MTC group, the increasing aberrant methylation of CpG74_a correlated with decreasing expression of the *Rap1Gap* gene ($R^2: 0.23$; $P=0.032$), while this correlation was not observed in the controls and benign nodule groups. The decreasing expression of the *RAP1GAP* gene was correlated with increasing aberrant methylation of the CpG24 in the MTC and benign groups ($R^2: 0.56$, $P=0.001$ and $R^2: 0.31$, $P=0.029$, respectively). The hyper-methylation of CpG74_b was not correlated with expression of the *RAP1GAP* gene ($P=0.234$).

Western Blotting Analysis

The western blotting results showed a low protein level of *RAP1GAP* in tissues from MTC patients compared to the control (0.85 ± 0.15 versus 3.7 ± 0.38 ; $P=0.017$). Benign nodules showed no obvious change in the expression of the *RAP1GAP* protein compared to the normal thyroid tissue (2.8 ± 0.31 versus 3.7 ± 0.38 ; $P=0.072$). The expression of the *RAP1GAP* protein in the MTC samples

Table 5. The Association of Methylation Status in Three CpGs with MTC

Models	CpG24	CpG74 _a	CpG74 _b
Model I	1.23 (0.21?1.85) $P=0.045$	1.34 (0.23?1.52) $P=0.046$	1.04 (0.15?1.12) $P=0.189$
Model II	1.65(0.75? 2.11) $P=0.022$	1.45 (0.32?1.25) $P=0.035$	1.15 (0.22? 2.1) $P=0.074$
Model III	1.28(0.15?1.42) $P=0.058$	1.40 (0.37?1.25) $P=0.083$	1.24 (0.19?2.89) $P=0.077$
Model IV	1.44(0.73?2.13) $P=0.063$	1.66 (0.52?1.83) $P=0.021$	1.31 (0.84?2.28) $P=0.58$
Model V	1.48(0.26?2.20) $P=0.088$	1.92 (0.67?2.31) $P<0.001$	1.58 (0.25?4.81) $P=0.032$

Model I, crude model; Model II, adjusted for sex and age; Model III, adjusted for sex, age and BMI; Model IV, adjusted for sex, age, T4 and TSH; Model V, adjusted for sex, age, calcitonin.

Control group was considered as reference in regression models. Multinomial logistic regression analysis was used to examine the effects of potential confounders.

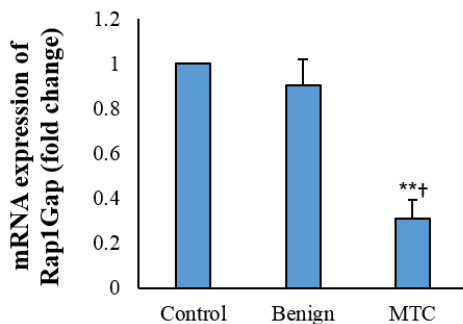


Figure 3. *Rap1Gap* mRNA Expression in Thyroid Tissues (Fold Change). Each assay was replicated 3 times, and presented as mean \pm SD. ** $P<0.01$, † $P<0.05$.

was considerably lower than the benign nodules (3.7 ± 0.38 versus 2.8 ± 0.31 ; $P=0.018$) (Figure 4).

Assessing the correlation of the *RAP1GAP* protein expression with the DNA methylation pattern of CpG74_a demonstrated that reduced expression of the *RAP1GAP* protein was considerably correlated with hyper-methylation in the MTC group ($R^2: 0.28$; $P=0.045$). However, this correlation was not seen in the benign and control groups. Expression of the *RAP1GAP* protein was also correlated with hyper-methylation rate of CpG24 in the benign nodules and MTC group ($R^2: 0.36$, $P=0.001$ and $R^2: 0.31$, $P=0.019$, respectively). The hyper-methylation rate of CpG74_b was not correlated with the *RAP1GAP* protein expression ($P=0.116$).

Discussion

This study showed that the expression of *RAP1GAP* as a suppressor gene in MTC patients was considerably lower than the normal individuals. Previous studies have also reported similar results for various tumor suppressor genes.¹³⁻¹⁵ Down-regulation of the *Rap1Gap* gene can be due to hyper-methylation of its regulatory region.¹² It has been reported that downregulation of *RAP1GAP* by promoter hyper-methylation promotes melanoma cell metastasis.¹⁶

To examine the relation of hyper-methylation to *RAP1GAP* gene expression, we evaluated the hyper-methylation pattern of three CpGs. Accordingly, all three CpGs were hyper-methylated in 31%, 22%, and 2% of tissues from patients with MTC, benign nodules, and normal thyroid groups, respectively. The hyper-methylation of two CpGs occurred in 69%, 41%, and 30% of tissues from patients with MTC, benign nodules, and normal thyroid. The hyper-methylation of only one CpG was seen in 9% of tissues from MTC, 32% of benign nodules, and 52% of the control group. Interestingly, the unmethylated pattern for all three CpGs was not observed

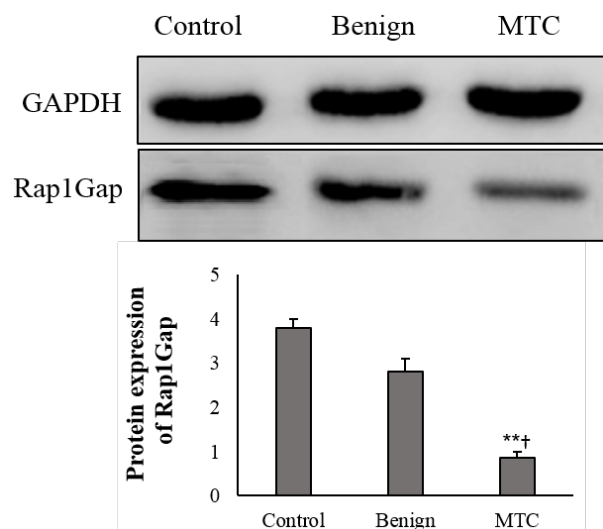


Figure 4. Expression of *RAP1GAP* Protein in Tissue Samples and Cells. The ImageJ software was used to quantify protein expression. The data were normalized to GAPDH band intensity. Each assay was done 5 times, and mean \pm SD is presented. ** $P<0.01$, † $P<0.05$.

in MTC tissues, indicating the methylation of CpGs to be associated with malignancy.

The methylation/unmethylation ratios of the CpG74_a and CpG24 were considerably high in the MTC patients. However, the methylation/unmethylation ratio of CpG24 was also high in the benign nodules. On the other hand, the methylation/unmethylation ratio of CpG74_b was not significantly greater than the controls. Therefore, CpG74_a can be considered as a specific epigenetic marker for clinical prognosis of MTC.

To determine the association of aberrant regulatory region methylation with expression of *RAP1GAP*, we compared the methylation status with the *RAP1GAP* expression data in MTC and normal thyroid tissues. In this regard, the decreasing mRNA expression and protein level of the *RAP1GAP* in the MTC patients were associated with the increasing methylation rate of the CpG74_a and CpG24.

The result of this study showed that in the MTC group, the hyper-methylation of CpG74_a was 92% higher than the normal thyroid group after adjustment for calcitonin. Calcitonin is an important MTC marker for prognosis, diagnosis, surgical planning, and follow-up.^{17,18} It has been demonstrated that serum levels of calcitonin correlate with tumor size and lymph node metastasis.¹⁸ A serum calcitonin level higher than 500 pg/mL was associated with lymph node metastasis.¹⁷ In line with these reports, in our study, the average calcitonin level of the MTC patients was 158 pg/mL. Interestingly, metastasis was not reported in the MTC group in this study.

There are limited studies that determine the importance of epigenetic variations, especially DNA methylation in the MTC. Ceolin et al indicated that global DNA methylation levels were higher in MTC compared to papillary thyroid cancer patients.² The role of epigenetics in MTC is largely defined as hyper-methylation of CpG sites in the promoter region of Ras-association domain family member 1A (*RASSF1A*) and telomerase reverse transcriptase (*TERT*) genes.¹⁹

In a large cohort study, Wang and colleagues reported that *TERT* gene hyper-methylation is related to high DNA copy numbers, and MTC patients with higher *TERT* methylation have lower chances of survival.²⁰

The results of a study in 2002 showed that *TERT* is aberrantly methylated in sporadic MTC. Additionally, it was found that increased methylation index was correlated with *TERT* gene expression.²¹ The hyper-methylation of CpG islands in the promoter region of *RASSF1A* have been reported in the MTC patients.²⁰⁻²²

Macia et al in 2012, showed that *Sporouty1* promoter is frequently hyper-methylated in MTC.¹⁴ CpG sites methylation of tumor-related promoters including *SLC5A8*, *RASSF1*, and *MGMT* occurs in undifferentiated thyroid carcinoma. Also, *SLC5A8* was the most noticeable hyper-methylated gene in thyroid cancers in a meta-analysis.¹⁹ In a study by Schagdarsurengin et al, *RASSF10* was hyper-methylated in 100% of MTC patients.²³

Conversely, CpG island methylation of *PTEN*, *MGMT*, *p16*, and *TSHR* genes have not shown any considerable hyper-methylation in the MTC individuals.²⁴⁻²⁶

Thyroid hormone synthesis-related genes are also epigenetically down-regulated during thyroid cancer promotion. The promoter region of the *TSH-R* gene was methylated in 25% of the thyroid cancer patients.²⁷

Various tumor-suppressor genes and thyroid hormone-related genes are epigenetically silenced in thyroid tumors.²⁸⁻³⁰ Epigenetic silencing has been highlighted in thyroid cancer progression. The promoter of the TSH receptor, pendrin, and NIS have been silenced in thyroid carcinomas.^{31,32}

In conclusion, this study identifies *RAP1GAP* as a candidate tumor-suppressor gene in MTC. Hyper-methylation in the regulatory region of the *RAP1GAP* gene may contribute to MTC pathogenesis and this status could be a new potential diagnostic biomarker.

Authors' Contribution

LK: supervised the study, data statistical analysis and critical reviewing of manuscript. BF: performed sampling, collection of data, experiments and data analysis. AAG: get the idea, supervised and data curation. MAG: designing the study, searching the literatures and, manuscript preparation. MT: performing clinical section and data acquisition. AA: definition of intellectual content and design the study. FA: made total concept, designing the study. HBJ: definition of intellectual content and design the study. AF: performing clinical section, data acquisition. MH: searching the literatures and manuscript editing.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interest.

Ethical Statement

This study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (No: Ajums. Rec.1393.151) and informed consent was provided by all participants included in the study.

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