Molecular and Cellular Pathobiology

Downregulation of Rap1GAP through Epigenetic Silencing and Loss of Heterozygosity Promotes Invasion and Progression of Thyroid Tumors

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Abstract

Thyroid cancer is the most common type of endocrine malignancy, encompassing tumors with various levels of invasive growth and aggressiveness. Rap1GAP, a Rap1 GTPase-activating protein, inhibits the RAS superfamily protein Rap1 by facilitating hydrolysis of GTP to GDP. In this study, we analyzed 197 thyroid tumor samples and showed that Rap1GAP was frequently lost or downregulated in various types of tumors, particularly in the most invasive and aggressive forms of thyroid cancer. The downregulation was due to promoter hypermethylation and/or loss of heterozygosity, found in the majority of thyroid tumors. Treatment with demethylating agent 5-aza-deoxycytidine and/or histone deacetylation inhibitor trichostatin A induced gene reexpression in thyroid cells. A genetic polymorphism, Y609C, was seen in 7% of thyroid tumors but was not related to gene downregulation. Loss of Rap1GAP expression correlated with tumor invasiveness but not with specific mutations activating the mitogen-activated protein kinase pathway. Rap1GAP downregulation was required in vitro for cell migration and Matrigel invasion. Recovery of Rap1GAP expression inhibited thyroid cell proliferation and colony formation. Overall, our findings indicate that epigenetic or genetic loss of Rap1GAP is very common in thyroid cancer, where these events are sufficient to promote cell proliferation and invasion. Cancer Res; 70(4); 1389–97. ©2010 AACR.

Introduction

Thyroid cancer is the most common type of endocrine malignancy and its incidence is rapidly growing in the United States and many other countries (1). Most of thyroid tumors originate from follicular epithelial cells and include benign follicular adenoma (FA), well-differentiated follicular carcinoma (FC) and papillary carcinoma (PC), and undifferentiated anaplastic carcinoma (AC; ref. 2). Patients with well-differentiated carcinomas usually have a good prognosis, but a subset of these cancers recurs and eventually causes patient death due to widely invasive local growth and metastasis to distant sites. Well-differentiated cancers may undergo de-differentiation and progression to AC, a formidable disease characterized by widespread invasion, early distant metastasis, and patient death within few months. The molecular mechanisms underlying proliferation, invasion, and progression of thyroid tumors are not fully understood (3).

PC, the most common type of thyroid cancer, accounts for ~80% of all thyroid malignancies. BRAF, RAS, and RET/PTC mutations, usually mutually exclusive in the same tumor, are found in ~70% of these tumors, indicating that RET/PTC–RAS–BRAF–mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK)–ERK or MAP kinase (MAPK) signal pathway plays a pivotal role in the initiation and progression of PC (4). However, other genetic events are very likely to exist and contribute to significant variation in gene expression profiling, phenotypical features, and biological characteristics seen among PCs (5, 6).

Rap1, a member of the RAS family of small GTPases, has been implicated in the regulation of mitogenic and oncogenic pathways in thyroid (7–9), and biochemical studies showed its role in regulating the ERK cascade and specifically its requirement for the RET/PTC-induced activation of BRAF–MEK–ERK (9–11). Like other small GTPases, Rap1 functions as a molecular switch, which cycles between an inactive GDP-bound and active GTP-bound form. Rap1GAP, a GTase-activating protein, functions as a negative regulator of Rap1 activity by facilitating hydrolysis of GTP to GDP. Recent findings suggest that Rap1GAP is frequently inactivated in several tumor types and may function as a tumor suppressor (10–13). However, its effects may vary in different cell types. In pancreatic cancer, Rap1GAP loss of heterozygosity (LOH) is frequently seen and loss of Rap1GAP function promotes growth, survival, and invasion of pancreatic cancer cells in vitro and in vivo (10). In squamous cell carcinoma (SCC) of the head and neck, Rap1GAP has been shown to inhibit cell proliferation (11) but promote invasion (12). In
thyroid tumors, expression of Rap1GAP protein has been found to be decreased in PCs when studied by immunohistochemistry (14), and in vitro Rap1GAP inhibited proliferation and invasion in thyroid cancer cell lines (15). Most recently, it has been shown that Rap1GAP is frequently downregulated in malignant melanoma via promoter hypermethylation, which promotes melanoma cell proliferation, survival, and migration (15). Rap1GAP alterations and expression have not been studied in various types of thyroid tumors, and molecular mechanisms responsible for downregulation of Rap1GAP in thyroid tumors remain largely unknown. In this study, we show that Rap1GAP expression is lost with progressively higher frequency in more aggressive types of thyroid cancer via promoter hypermethylation and LOH, and this plays an important role in promoting invasion of thyroid cancer cells.

Materials and Methods

Human tissue samples and cell lines. We analyzed 204 snap-frozen thyroid samples collected using the University of Pittsburgh Institutional Review Board (IRB)–approved protocols. They included 7 normal thyroid samples, 40 hyperplastic nodules (HN), 49 FAs, 27 FCs, 78 PCs, and 3 ACs. In addition, 92 neonatal cord blood samples from live births at Magee-Womens Hospital in Pittsburgh were used as a source of genomic DNA from unscreened, population-based controls. In accordance with University of Pittsburgh IRB regulations, no information apart from ethnicity was available for these samples. All the samples were obtained from samples with Caucasian ancestry (maternal report). All thyroid cell lines were obtained from Dr. James Fagin (Memorial Sloan-Kettering Cancer Center) in 2003 to 2007. The cell lines were tested and authenticated in October 2008 in the Molecular Pathology Laboratory at the University of Pittsburgh Medical Center using the ampFLSTR Identifiler PCR Amplification kit (Applied Biosystems), which tests for 16 different polymorphic loci. All cell lines tested had unique genetic profiles, and TPC1, Hth74, and TTA1 cell lines matched the size of alleles reported by Schweppe and colleagues (16).

Quantitative reverse transcription-PCR. To measure Rap1GAP mRNA expression, quantitative reverse transcription-PCR (qRT-PCR) was performed on ABI Prism 7500 (Applied Biosystems). Rap1GAP expression was assessed using SYBR Green (Applied Biosystems) and primers 5′-AGGACATGTCATCAGCAAT-3′ (forward) and 5′-ACGAGCGGTGTATCC-3′ (reverse). Amplification of gliceraldehyde-3-phosphate dehydrogenase was used for normalization. The analysis was performed in duplicate. The Rap1GAP relative expression levels in tumor samples were calculated using the comparative Ct method, with the expression level averaged from seven normal thyroid tissues set as 1.0.

Detection of Rap1GAP genetic variations. Alterations in Rap1GAP gene were detected by direct sequencing and by LightCycler PCR followed with fluorescence melting curve analysis (FMCA). Direct sequencing of the coding sequence of Rap1GAP was performed from RNA following PCR amplification with four pairs of primers. For genomic DNA and LightCycler PCR, two pairs of primers flanking possible mutation sites 769T>C (C257R) and 1826A>G (Y609C) were used for amplification. For position 1826, a pair of primers flanking the mutation site was used (5′-AAGGTAGCGCTGTTTG-3′ and 5′-AGGAGACGGTGTATCC-3′), together with two fluorescent probes (sensor 5′-GGGACTCCTTCATCTATAGCAGC-3′ and anchor 5′-LC Red-GGCTGGAGGACGTCGACGAC-3′).

Detection of BRAF and RAS mutations and RET/PTC rearrangements. V600E BRAF mutation was detected by real-time PCR and FMCA from DNA as previously reported (17). Point mutations of the RAS gene family most commonly found in thyroid cancer, NRAS codon 61, HRAS codon 61, and KRAS codon 12/13, were detected from DNA using PCR and FMCA on LightCycler as previously reported (18). Two main types of RET/PTC rearrangement, RET/PTC1 and RET/PTC3, were detected from DNA by RT-PCR, with primers flanking the respective fusion point, followed by agarose gel electrophoresis of the PCR products as previously reported (19).

Immunohistochemistry. Immunohistochemistry for Rap1GAP was performed on formalin-fixed, paraffin-embedded tissues using avidin-biotin complex method. Primary Rap1GAP antibody (Santa Cruz Biotechnology) was used at 1:100 dilution with overnight incubation at 4°C. The staining intensity of staining was at least 50% weaker as compared with the adjacent nonneoplastic thyroid cells.

LOH analysis. LOH in the Rap1GAP region was studied using PCR amplification and capillary gel electrophoresis for two microsatellite loci (RAPGA1 and D1S2828) located on 1p36.1-p35 within the Rap1GAP gene.

Methylation-specific PCR. Two CpG islands within Rap1GAP promoter region, CpG24 and CpG74, contain 24 and 74 CpG individual sites, respectively (Supplementary Fig. S1). For methylation-specific PCR analysis, CpG24 island and two CpG units within CpG74 island close to transcription start site were selected. Three sets of methylation-specific and not methylation-specific primers were designed for CpG24, CpG74A, and CpG74B using MethPrimer (20). Before amplification, DNA was subjected to bisulfite treatment using EpiTect Bisulfite kit (Qiagen) following the manufacturer’s instructions. Universal Methylated Human DNA Standard (Zymo Research Corp.), enzymatically methylated human genomic DNA by SsoI methylase, was used as a positive control.

5-Aza-deoxycytidine and trichostatin A treatment. DNA demethylating agent 5-aza-deoxycytidine (5-Aza) and histone deacetylase inhibitor trichostatin A (TSA) were purchased from Sigma Chemical Co. TPC1 and Hth83 cells were plated in 24-well plate and grown for 24 h before treatment. 5-Aza was added in concentrations of 5, 15, and 25 μmol/L for 72-h treatment. TSA was added in concentration of 1 μmol/L for only 24-h treatment, either alone or in combination with 5 μmol/L 5-Aza, after cells have been treated...
with 5-Aza alone for 48 h. As control, cells were maintained in the regular medium without drug addition. Cells were harvested after treatment; protein was used for analysis of Rap1GAP expression and DNA was isolated for methylation-specific PCR.

**Western blot.** Protein extracts were prepared from frozen tissue using T-PER Tissue Protein Extraction Reagent (Pierce) and from culture cells using Lysis-M Reagent (Roche Applied Science) with protease inhibitors (Complete, Mini, EDTA-free Protease Inhibitor Cocktail tablets, Roche). Samples were subjected to SDS-PAGE and immunoblotted with anti-Rap1GAP (Santa Cruz Biotechnology) antibodies.

**Site-directed mutagenesis.** Hemagglutinin (HA)-Rap1GAP609mut mutagenesis was done using the QuikChange Site-Directed Mutagenesis kit (Stratagene) using standard-purified mutagenesis primers (designed with the QuikChange Primer Design Program) and wtRap1GAP plasmid (kindly provided by Dr. Stork, Oregon Health Sciences University, Portland, OR) as DNA template following the manufacturer’s recommendations. Mutant sequence was then swapped into pm12SM-HA-Rap1GAP vector (provided by Dr. J. Bos, Utrecht University Medical Center, Utrecht, the Netherlands).

**Rap1 activation assay (RalGDS-RBD pull down).** HEK293 cells (~3 × 10⁵ per well in six-well plates) were transfected with 3 μg of total DNA and 6 μg PEI (Polysciences). HA-Rap1b (250 ng) was cotransfected with myc-Epac (500 ng) and HA-Rap1GAP wild-type (WT) or Y690C (1–2.25 μg). After 24 h, cells were serum starved for 2 h followed by stimulation with forskolin (20 μmol/L). Cells were rinsed with cold PBS and lysed in buffer containing 200 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 1% NP40, 10% glycerol, 25 mmol/L MgCl₂, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μmol/L leupeptin, and 2 μmol/L aprotinin. Lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4°C, and 10 μg of bacterially expressed glutathione S-transferase–RalGDS–RBD coupled to glutathione–Sepharose beads (Amersham Biosciences) were added to the supernatants. On 60-min incubation at 4°C, the beads were washed four times in the same lysis buffer. After the final wash, Laemmli sample buffer was added to the samples. Proteins were fractionated in a 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Western blots were performed with anti-HA antibody (Covance).

**Cell proliferation, tumorigenicity, and invasiveness assays.** Transient transfection of Rap1GAP and control plasmids was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. For proliferation assay, cells were harvested 24 h after transfection and seeded at 1 × 10⁴ per well in 96-well plates. Cell proliferation was measured using the Rapid Cell Proliferation Kit (Calbiochem) for 3 d after plating. All samples were assayed in triplicate. For colony formation assay, cells were harvested after transfection and seeded at 1 × 10³ per dish in 10-cm dishes in triplicate. The cells were cultured in growth medium containing 400 μg/mL G418. After 14 d, colonies were stained with 0.25% crystal violet and counted. For in vitro cell invasion assay, cells were harvested after transfection and plated in RPMI 1640 with 1% fetal bovine serum (FBS) at a density of 10⁵ per well in the upper chamber of BioCoat Matrigel Invasion 24-well plate chambers (BD Biosciences), containing 8-μm pore size filter, and exposed to RPMI 1640 with 10% FBS in the lower chamber. The invasion rate was evaluated after 22 h according to the manufacturer’s instructions. The assay was performed in triplicate. For wound-healing assay, cells transfected with Rap1GAP or vector plasmid were scraped with a pipette tip 48 h later and washed with PBS to remove the floating cells. Photographs were taken at 0- and 23-h time points.

**Statistical analysis.** One-way ANOVA (post hoc Bonferroni’s test) was used to analyze the expression data for Rap1GAP in clinical cases of thyroid tumors, and Mann-Whitney U test was used to analyze data from LOH screening. Student’s t test (unpaired, two-tailed) was used to analyze data from colony formation, invasion, wound-healing, and proliferation assays. Fisher’s exact test (two-sided) was used to analyze data from immunohistochemistry. All values were represented as mean ± SD. Significance was determined at P < 0.05.

## Results

**Rap1GAP Is Significantly Downregulated in Thyroid Tumors**

The expression of Rap1GAP mRNA was studied in 15 normal thyroid tissues and 197 samples that represent the entire spectrum of follicular cell–derived thyroid tumors and nonneoplastic nodular lesions, including benign HN and FA, well-differentiated PC and FC, and undifferentiated AC. qRT-PCR results revealed that, as compared with normal thyroid tissue, Rap1GAP mRNA levels were maintained in HNs (relative expression, 0.94) but were progressively lower in benign and particularly malignant tumors (Fig. 1A). In benign FAs, the decrease was not statistically significant (0.71; P = 0.07), whereas Rap1GAP levels were significantly downregulated in all malignant tumors, including FAs (0.46), PCs (0.25), and ACs (0.04; P < 0.01).

To determine whether there was a downregulation of Rap1GAP at the protein level, 15 normal thyroid tissues and 105 tumor samples were studied by immunohistochemistry. Using semiquantitative assessment of the immunostain, a decrease or complete loss of Rap1GAP expression was observed in 0 of 28 HNs, 2 of 32 (6%) FAs, 6 of 16 (38%) FCs, and 21 of 29 (72%) PCs (P < 0.001; Fig. 1B). To further verify the loss of Rap1GAP protein in thyroid tumors, three PCs, which showed downregulation of mRNA and loss of protein expression by immunohistochemistry, were studied by Western blotting. It confirmed the markedly decreased levels of Rap1GAP protein in all of these PCs as compared with the paired normal thyroid tissues (Fig. 1C).

Similar findings were obtained in thyroid cell lines (Fig. 1D). Rap1GAP expression was preserved in FRTL5 and PCCl3 cells, both of which are nontransformed rat thyroid cell lines, and was lost or markedly decreased in four of six cancer cell lines, including TTA1, Hth83, Hth74, and TPC1 cells, which are derived from human ACs (TTA1, Hth83, and Hth74) and PCs (TPC1). However, WRO and ARO cells...
showed preserved Rap1GAP expression. Whereas WRO cells are derived from thyroid FC, ARO cells, previously believed to be derived from thyroid AC, have been recently shown to be of nonthyroid origin (16).

The qRT-PCR results for Rap1GAP expression were correlated with the mutational status in 65 PCs. No difference between Rap1GAP expression levels and BRAF, RAS, or RET/PTC mutations was found (data not shown). This suggests that Rap1GAP downregulation, which is very common in PCs and other types of thyroid cancer, is not directly related to the activation of specific effectors of the MAPK signaling cascade.

**Mechanisms of Rap1GAP Downregulation in Thyroid Tumors**

To identify the mechanisms responsible for Rap1GAP downregulation in thyroid tumors, the tumor samples were tested for mutations and other genetic variations in the Rap1GAP gene, LOH in this region, and promoter hypermethylation.

**Genetic variations in the Rap1GAP gene.** First, the entire open reading frame of the Rap1GAP gene was analyzed in 12 PCs by direct sequencing. A heterozygous alternation (1826A>G) leading to Y609C was found in one tumor. No other alterations were identified. The Y609C variant as well as Rap1GAP C257R were two mutations previously reported in breast cancer (21). Then, we screened DNA from additional 45 cases of thyroid tumors for these two putative mutations by direct sequencing. Three additional Y609C alterations were found. We further studied the prevalence of Y609C alteration in 136 samples using LightCycler real-time PCR and FMCA. Totally, 151 thyroid tumors were screened and 10 (6.8%) were found to harbor a heterozygous Y609C allele (Table 1). Analysis of normal tissue in three cases with tumors positive for Y609C also identified Y609C in normal tissue, indicating that this was a germline genetic event.

To assess whether Y609C may affect Rap1GAP catalytic activity, we monitored Rap1-GTP by RalGDS-RBD pull-down assay. cAMP-Epac was used to increase Rap1-GTP levels, and hydrolytic activities of WT and Y609C Rap1GAP were compared. As shown in Fig. 2, both GAP proteins downregulated Rap GTP in a dose-dependent manner, indicating no effect of Y609C on Rap1GAP activity.

To find if Rap1GAP Y609C is a rare mutation or if it represents a common single nucleotide polymorphism (SNP), the frequency of this variant was evaluated in 92 genomic DNA samples from population-based controls (184 chromosomes). Two individuals were heterozygous for Y609C. Thus, the frequency of the G allele (Y609C) was 1.1%, indicating that Rap1GAP Y609C is probably a SNP. The frequency of this allele was somewhat greater among patients with thyroid tumors (3.4%), but the difference was not statistically significant (P = 0.145).

**Loss of heterozygosity.** The LOH status of this region was tested in 135 thyroid tumors using two microsatellite markers.
Table 1. Prevalence of Rap1GAP Y609C variant in patients with various thyroid tumors and benign nodules and in the general population

<table>
<thead>
<tr>
<th>No. cases examined</th>
<th>Rap1GAP Y609C (%)</th>
<th>Prevalence of Y609C allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN</td>
<td>42</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Thyroid tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>49</td>
<td>3 (6.1%)</td>
</tr>
<tr>
<td>FC</td>
<td>34</td>
<td>3 (8.8%)</td>
</tr>
<tr>
<td>PC</td>
<td>65</td>
<td>3 (4.6%)</td>
</tr>
<tr>
<td>AC</td>
<td>3</td>
<td>1 (33.3%)</td>
</tr>
<tr>
<td>General population</td>
<td>92</td>
<td>2 (2.1%)</td>
</tr>
</tbody>
</table>

(Rap1GA1 and D1S2828) located within the Rap1GAP. The analysis was informative in 128 tumors, and 37 (29%) of them showed LOH in at least one of those two loci (Supplementary Table S1). The frequency of LOH was 16% in FA, 21% in FC, 41% in PC, and 67% in AC. A significant correlation between LOH and decreased Rap1GAP mRNA expression level was found ($P = 0.03$, Mann-Whitney U test), indicating that LOH is likely to be responsible for the decreased expression of Rap1GAP in a subset of thyroid tumors.

Promoter methylation. Two known CpG islands within Rap1GAP promoter region, CpG24 and CpG74, contain 24 and 74 individual CpG units, respectively (Supplementary Fig. S1). For methylation analysis of Rap1GAP promoter region, CpG24 island and two individual CpG units within CpG74 island closest to the transcription start site (CpG74A and CpG74B) were used. Methylation status of these three CpG areas was analyzed in 58 PCs. Whereas 17 (29%) tumors were found unmethylated at these sites, 26 (45%) revealed methylation at one site, 10 (17%) at two sites, and 5 (9%) at all three sites. The presence and extent of methylation of CpG islands in the promoter region showed a strong inverse correlation with Rap1GAP expression at the mRNA level ($r^2 = 0.9993$), with tumors hypermethylated in all three sites showing the lowest levels of Rap1GAP expression (Fig. 3A). As a control, methylation status was also examined in five normal thyroid tissues obtained after thyroidectomy and showed no methylation at most of these sites (Supplementary Fig. S2).

The combined results of methylation and LOH analyses of 53 PCs showed that in this group of tumors, 7 (13%) had LOH, 26 (45%) had methylation, 15 (28%) had both LOH and methylation, and 7 (13%) had neither LOH nor methylation, showing the presence of one or both of these alterations in the majority of human PCs.

Next, we examined the methylation status of these CpG sites in thyroid cancer cell lines. Hypermethylation of all three CpG sites in the Rap1GAP promoter region was detected in Hth74, Hth83, TPC1, and TTA1 cells (Fig. 3B). Importantly, all of them had no Rap1GAP expression as shown earlier by Western blot (Fig. 1D). In ARO cells that preserved Rap1GAP expression, none of those three CpG sites was methylated, and in WRO cells with slightly decreased Rap1GAP expression, only CpG24 was methylated.

To confirm that epigenetic mechanisms cause decreased Rap1GAP expression in thyroid cancer cells, we treated TPC1 and Hth83 cells with 5-Aza and TSA to find whether demethylation of Rap1GAP promoter and/or acetylating histones could induce reexpression of Rap1GAP. In both cell lines, treatment with 5-Aza (5–25 μmol/L) resulted in various levels of restoration of Rap1GAP expression (Fig. 3C and D). In Hth83 cells, 5-Aza treatment induced Rap1GAP reexpression in a dose-dependent manner and in correlation with alteration of methylation status. Complete demethylation achieved by 25 μmol/L 5-Aza resulted in the highest levels of Rap1GAP expression. In TPC1 cells, 5-Aza treatment resulted in modest increase in Rap1GAP expression.

However, TSA induced pronounced restoration of Rap1GAP expression in TPC1 cells. In Hth83 cells, treatment with TSA had no effect on Rap1GAP expression. These results provide direct evidence that epigenetic mechanisms underlie downregulation of Rap1GAP expression in many thyroid tumors.

Correlation between Rap1GAP Loss and Thyroid Tumor Invasiveness

In the mRNA and immunohistochemical studies described above, loss or reduction in Rap1GAP expression generally correlated with the degree of invasiveness of specific tumor.
types, being lowest in PCs and ACs, which have the most widespread infiltrative growth. To assess the in vivo effects of loss of Rap1GAP on invasiveness in thyroid tumors more accurately, we evaluated the expression levels of Rap1GAP by immunohistochemistry in 49 PCs and 20 FCs, in which tumors were grouped by invasiveness. Among PCs, the decreased or loss of Rap1GAP immunostaining was observed in 28 of 29 (97%) invasive PCs but only in 4 of 20 (20%) encapsulated PCs ($P < 0.001$). Among FCs, the decrease or loss of immunostaining was found in 5 of 5 (100%) widely invasive FCs and in 2 of 15 (13%) minimally invasive FCs ($P = 0.001$).

The role of loss of Rap1GAP in invasiveness of thyroid cells was further confirmed in vitro. Reexpression of Rap1GAP in TPC1 and Hth83 thyroid cells led to inhibition of Matrigel invasion and decreased migration ability in the wound-healing assay (Fig. 4).

Recovery of Rap1GAP Expression Inhibits Thyroid Cancer Cell Growth In vitro

To examine whether Rap1GAP may serve as a potential therapeutic target, we studied the effect of restoration of Rap1GAP expression on thyroid tumors more accurately, we evaluated the expression levels of Rap1GAP by immunohistochemistry in 49 PCs and 20 FCs, in which tumors were grouped by invasiveness. Among PCs, the decreased or loss of Rap1GAP immunostaining was observed in 28 of 29 (97%) invasive PCs but only in 4 of 20 (20%) encapsulated PCs ($P < 0.001$). Among FCs, the decrease or loss of immunostaining was found in 5 of 5 (100%) widely invasive FCs and in 2 of 15 (13%) minimally invasive FCs ($P = 0.001$). The role of loss of Rap1GAP in invasiveness of thyroid cells was further confirmed in vitro. Reexpression of Rap1GAP in TPC1 and Hth83 thyroid cells led to inhibition of Matrigel invasion and decreased migration ability in the wound-healing assay (Fig. 4).

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Discussion

In this study, we show that Rap1GAP has important tumor suppressor functions in thyroid cells and is frequently lost through epigenetic and genetic mechanisms in most aggressive forms of thyroid cancer.

Common mechanisms of gene silencing and inactivation of tumor suppressor genes include inactivating mutations, LOH, and epigenetic alterations. Our results showed that the major mechanisms of Rap1GAP inactivation in thyroid cells are LOH and hypermethylation of the promoter region. Rap1GAP is located at human chromosome 1p36.1-p35, a region in which high frequency of LOH has been reported in several types of human cancer, including pancreatic carcinoma and oral SCC. A recent study of pancreatic cancers showed a 33% frequency of LOH in the Rap1GAP gene region in these tumors (10). It seems that this mechanism is also common in thyroid cancer, as we observed high frequency
of LOH in this region, particularly in most invasive tumor types.

Another mechanism of Rap1GAP identified in this study was through hypermethylation of CpG islands in the promoter region of the gene. The extent of hypermethylation correlated with expression levels of Rap1GAP. Moreover, treatment with 5-Aza and/or TSA in thyroid cancer cell lines with depleted Rap1GAP expression and hypermethylation in promoter restored the expressions of Rap1GAP. During the preparation of the manuscript, downregulation of Rap1GAP via promoter hypermethylation was identified in melanoma (15). Because epigenetic gene silencing through promoter methylation and changes in chromatin structure is reversible, our data suggest that Rap1GAP may serve as a potential target for thyroid cancer therapy. This would be of particular importance for anaplastic thyroid carcinoma, which is one of the most aggressive human tumors with short survival and lack of effective therapeutic strategies.

Our data indicate that in thyroid cells, Rap1GAP loss is important for tumor cell invasion. Strong correlation with tumor invasiveness was observed in the most common types of thyroid cancer (i.e., PCs and FCs), as in both tumor types the loss of Rap1GAP was largely limited to the tumor with extensive invasion. Similarly, expression of Rap1GAP in thyroid cells in vitro significantly inhibited cell migration and invasion. Similar results have been recently shown in human follicular thyroid carcinoma FTC-133 cells (13) and in pancreatic cancer cells (10). However, in oropharyngeal SCCs, Rap1GAP seems to have an opposite effect on invasion, as it has been shown to promote invasion via induction of matrix metalloproteinase (MMP)-9 secretion, which is associated with poor survival in low N-stage SCC (12). The reasons for different effect of Rap1GAP on tumor invasiveness in oropharyngeal SCC as compared with pancreatic and thyroid cancer are not clear. It is conceivable that this may be due to tissue-specific effects of Rap1GAP expression on MMP production. In thyroid cancer cells, a link between enhanced productions of MMPs and BRAF mutation has been found, which may help to explain the more invasive behavior of thyroid cancers carrying BRAF mutation (22). However, gene expression array data revealed no significant association between Rap1GAP expression and expression of MMP-2, MMP-3, MMP-9, and MMP-13 in thyroid PCs (5).

Figure 4. Reexpression of Rap1GAP inhibits invasion and migration of human thyroid carcinoma cells. A, Rap1GAP significantly impaired the ability of TPC1 and Hth83 cells to invade through Matrigel. The percentage of cells that penetrated through Matrigel-coated Transwell chambers is shown based on three experimental replicates. B, wound-healing assay. Confluent Hth83 and TPC1 cells transfected with either Rap1GAP or vector plasmid for 48 h were wounded, and images were acquired immediately and 23 h later.
We also observed that restoration of Rap1GAP inhibits thyroid tumor cell proliferation and growth. These findings corroborate the results recently reported in pancreatic cancer, where Rap1GAP expression suppresses tumor formation and progression in vitro and in vivo and also increases apoptotic rates of cancer cells in response to chemotherapeutic drugs 5-fluorouracil and etoposide (10). In oropharyngeal SCC, Rap1GAP expression inhibits tumor growth in vivo (11). These findings provide additional evidence for the role of Rap1GAP downregulation in tumor growth and invasion in thyroid and other cell types and suggest that restoration of Rap1GAP expression may be exploited as a potential therapeutic approach for these tumors.

Two genetic alterations in the Rap1GAP gene (C257R and Y609C) were previously identified in breast cancer, and those believed to be mutations (21); these genetic variants have not been listed in most SNP databases. However, the results of our study indicate that the Y609C allele is present with 1.1% incidence in the general population and therefore likely represents a SNP. The incidence of this allelic variant is 3.4% in patients with thyroid tumors, as detected in a series of 151 patients. The difference is not statistically significant; however, whether this genetic variation is associated with predisposition to thyroid tumors will require the analysis of a much larger cohort of patients. It is unlikely that Y609C directly affects the function of the gene, as the Y609C Rap1GAP revealed no effect on Rap1 activity in the pull-down assay as compared with WT Rap1GAP. This finding is not unexpected because Y609C is located outside of the core of Rap1GAP protein, which is considered to be essential for its function. Indeed, it has been shown that of 663 amino acid residues, only amino acids 75 to 416 are required for full GAP activity (23).

In summary, the results of this study show that Rap1GAP is likely to serve as an important tumor suppressor in thyroid cells and its loss during carcinogenesis contributes to tumor progression and invasion. Restoration of Rap1GAP expression by releasing the epigenetic block or by other mechanisms has an inhibitory effect on invasion and proliferation of thyroid cancer cells, suggesting that Rap1GAP may serve
as a novel therapeutic target for most aggressive types of thyroid cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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