Early Occurrence of RASSF1A Hypermethylation and Its Mutual Exclusion with BRAF Mutation in Thyroid Tumorigenesis

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ABSTRACT

Follicular epithelial cell-derived thyroid tumors are common neoplasms comprised mainly of benign thyroid adenomas, follicular thyroid cancers, and papillary thyroid cancers (PTCs). Hypermethylation of the tumor suppressor gene RASSF1A and activating mutation of BRAF gene have been reported recently in thyroid cancers. To additionally investigate the roles of these two epigenetic/genetic alterations in thyroid tumor progression, we examined their occurrences and relationship in both benign and malignant thyroid neoplasms. With real-time quantitative methylation-specific PCR, we found that 4 of 9 (44%) benign adenomas, 9 of 12 (75%) follicular thyroid cancers tumors, and 6 of 30 (20%) of PTC tumors harbored promoter methylation in ≥25% of RASSF1A alleles. Additional quantitative analysis revealed RASSF1A methylation only in BRAF mutation-negative PTCs. A similar inverse correlation of RASSF1A methylation with BRAF mutation was seen in thyroid tumor cell lines. Our results, therefore, suggest that epigenetic inactivation of RASSF1A through aberrant methylation is an early step in thyroid tumorigenesis. Like the previously reported mutually exclusive relationship between BRAF mutation and other Ras pathway components such as RET/PTC rearrangement, a mutually exclusive relationship also exists between BRAF mutation and RASSF1A methylation in thyroid tumorigenesis.

INTRODUCTION

Thyroid tumors derived from follicular epithelium are common neoplasms, accounting for the majority of thyroid nodules found in about 30–60% of autopsies and 5% of the normal population on routine physical examination (1). Histologically, these tumors represent mostly benign adenomas and less frequently follicular and papillary thyroid carcinomas (FTC and PTC, respectively). Genetic alterations are common in thyroid cancers, including BRAF gene mutation (2–7) and RET/PTC rearrangements (8) in PTC, and Ras proto-oncogene mutations (8) and oncogenic PAX8-PPARγ fusions (9) in FTC. Genetic alterations may also lead to the development of benign thyroid adenomas and their possible malignant transformation, as suggested by the increased incidence of benign adenoma after thyroid irradiation exposure (10), and by the clonal nature of adenoma (11, 12). Activating point mutations of the thyroid stimulating hormone receptor gene are frequently found in autonomous benign thyroid adenomas (13, 14). However, clinical consensus data showed little probability of malignant transformation of these benign tumors (15). Some classic oncogenic genetic alterations, such as Ras mutation, occur in some benign thyroid adenomas, but their specific role is unclear in these benign neoplasms (8), although Ras mutation was shown recently to be associated with more aggressiveness and poor prognosis in thyroid cancer (16). Moreover, whether FTC or PTC occurs de novo or develops from benign thyroid adenomas has not been established, although, because of histological similarities, FTC is proposed to arise from adenomas (1, 8).

Epigenetic alterations of genes, such as aberrant promoter methylation, are common and important mechanisms involved in tumorigenesis (17), and may be alternative mechanisms to gene mutations for the formation of thyroid cancers. An example is the tumor suppressor gene RASSF1A, which is ubiquitously expressed in normal tissues and silenced in numerous cancers through promoter hypermethylation (18, 19). RASSF1A hypermethylation was also reported recently in thyroid cancers (20). However, in this study, using conventional methylation-specific PCR (MSP), RASSF1A methylation was not examined in benign thyroid neoplasms and was not compared with common genetic alterations such as BRAF mutation. Consequently, we used quantitative real-time MSP, and investigated the methylation status of RASSF1A and the clonal expansion of cells bearing this epigenetic change in various thyroid neoplasms including benign adenomas. We observed frequent early aberrant methylation of RASSF1A in adenomas and a significant clonal expansion of this methylation (with >30% of the tumors having nearly 100% allelic methylation) in FTC and BRAF mutation-negative PTC. We thus demonstrated an early role of RASSF1A methylation and its inverse relationship with BRAF mutation in thyroid tumorigenesis.

MATERIALS AND METHODS

Human Thyroid Tissues, Human Thyroid Tumor Cell Lines, and DNA Isolation. Human thyroid tissues were obtained and microdissected from fresh surgical samples or paraffin-embedded blocks as described previously (21). The study was approved by the Joint Committee on Clinical Investigation of the Johns Hopkins University School of Medicine. Collection of the tumor samples from Yale University School of Medicine (through Drs. Giovanni Tallini and Robert Udelsman) was approved by the local Institutional Review Board. Thyroid tumor cell lines were kindly provided by the following investigators, Dr. Kenneth B. Ain (University of Kentucky Medical Center, Lexington, KY) for KAK-1, KAT-5, KAT-7, and KAT-10; Dr. Guy J. F. Juillard (University of California Los Angeles School of Medicine, Los Angeles, CA) for ARO-81–1; and Dr. Nils-Erik Heldin (University of Uppsala, Uppsala, Sweden) for C643. These cell lines were cultured at 37°C in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% calf serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and penicillin-streptomycin, in a humidified incubator with 5% CO2.

DNA isolation was performed as described previously (21). Briefly, fresh tumor tissues were subjected to digestion with 1% SDS and 0.5 mg/ml proteinase K at 48°C for 48 h, with the addition of a spiking dose of concentrated SDS/proteinase K in the middle of this digestion period. For paraffin-embedded tissues, before proteinase K digestion samples were first treated with xylene for 8 h at 48°C to remove the paraffin. DNA was

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Notes: M. Xing, Y. Cohen, and E. Mambo contributed equally to this work. Under a licensing agreement between OncoMethylome Sciences, SA and the Johns Hopkins University, D. Sidransky is entitled to a share of royalty received by the University on sales of products described in this article. D. Sidransky owns OncoMethylome Sciences, SA stock, which is subject to certain restrictions under University policy. D. Sidransky is a paid consultant to OncoMethylome Sciences, SA and is a paid member of the company’s Scientific Advisory Board. The term of this arrangement is being managed by the Johns Hopkins University in accordance with its conflict of interest policies. Present address for G. Tallini: Anatomia Patologica, Ospedale Bellaria, Via Altura 3, 40139 Bologna, Italy.

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Table 1  Real-time quantitative methylation specific PCR analysis of RASSF1A methylation in thyroid tumors

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<tr>
<th>Tumor types</th>
<th>Methlated/total</th>
<th>%</th>
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<tbody>
<tr>
<td>Normal thyroid</td>
<td>0/14</td>
<td>0</td>
</tr>
<tr>
<td>Benign adenoma</td>
<td>4/9</td>
<td>44</td>
</tr>
<tr>
<td>Follicular cancer</td>
<td>9/12</td>
<td>75</td>
</tr>
<tr>
<td>Papillary cancer</td>
<td>6/30</td>
<td>20</td>
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* The data are presented as the number and percentage of cases for each type of thyroid tumor that reached a level of RASSF1A methylation of ≥25% of the total alleles.

subsequently isolated from the digested tissues by phenol-chloroform extraction and ethanol precipitation, and resuspended in H2O for final use. DNA isolation from cell lines was similarly performed as described (21).

**Bisulfite Treatment of DNA.** DNA was treated with sodium bisulfite to convert cytosine to uracil as described previously with slight modification (22). Briefly, a final volume of 22 μl mixture in H2O containing ~2 μg DNA, 5 μg salmon sperm DNA, and 0.3 μl NaOH was incubated at 50°C for 20 min to denature the DNA. The DNA was then incubated for 3 h at 70°C in a 500-μl reaction mixture containing 0.125 M hydroquinone and 2.5 M sodium metabsulfite (pH 5.0). After this treatment, the DNA was purified using the Wizard DNA purification system following the instructions of the manufacturer (Promega Corp., Madison, WI), followed by ethanol precipitation, vacuum drying, and resuspension in 20 μl H2O.

**Real-Time Quantitative MSP.** Real-time quantitative MSP was performed as described previously (22) with some modifications. Briefly, the reactions were performed in triplicate for each DNA sample in a total volume of 20 μl containing 3 μl of bisulfite-treated genomic DNA, 16.6 mM ammonium sulfate, 67 mM Trizma, 2.5 mM MgCl2, 10 mM β-mercaptoethanol, 0.1% DMSO, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 600 nM each of sense and antisense primers, 200 μM TaqMan probe, 0.6 units platinum Taq polymerase, and 2% Rox reference dye. After an initial denaturation step at 95°C for 2 min, 50 cycles of 15 s at 95°C and 60 s at 60°C for annealing and extension were run using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The cycling conditions were optimized with native lymphocyte DNA as a negative control and methyltransferase-treated lymphocyte DNA as a positive control. The primers and probe used for RASSF1A gene were as described previously for RASSF1A real-time quantitative MSP (23) and were: 5' CCT GTA CTT CGC TAA CTT TAA ACG 3' (sense); 5' CCC GTA CTT CGT TAA CTT TAA AGC 3' (antisense); and 5' FAM-ACA AAC GCC GAC CGA ACG AAA CCA-TAMRA 3' (probe). The primers and probe for the reference gene β-actin were 5' TGG TGA TGG AGG TTT AGT AAG T 3' (sense); 5' AAC CAA TAA AAC CTA CTC CTC CTT TAA 3' (antisense); and 5' FAM-ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA-TAMRA 3' (probe). The relative degree of methylation of each DNA sample (as % of the total alleles) was calculated using the method described previously (24).

**BRAF Mutation Analysis.** The BRAF T1796A transversion mutation in exon 15 of the BRAF gene was analyzed by direct DNA sequencing. Briefly, a 212-bp fragment from exon 15 of the BRAF gene containing the site where the T1796A transversion mutation occurs was amplified by PCR using genomic DNA as templates and the primers as described (25): 5’ TCATAATGCTT-GCTCTGATAGGA 3’ (sense) and 5’ GGCCAAAAATTTAATCGTGG 3’ (antisense). The PCR conditions were: 5% DMSO, 16.6 mM ammonium sulfate, 6.7 mM MgCl2, 10 mM β-mercaptoethanol, 1.67 μM each primers, 1.5 mM each deoxynucleotide triphosphate, 0.5 unit of platinum DNA Taq polymerase (Life Technologies, Inc.), 67 mM Tris (pH 8.8), and about 60 ng genomic DNA in a 30-μl final volume. A step-down PCR protocol was used to ensure the specificity: 95°C for 5 min x 1 cycle; 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min x 2 cycles; 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min x 2 cycles; 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min x 35 cycles; with a final extension at 72°C for 5 min. The PCR products were subsequently subjected to direct sequencing PCR reaction with the sense primer and Big Dye terminator cycle sequencing reagents (Applied Biosystems) at the PCR settings of 95°C for 30 s, ×11 cycle; 95°C for 15 s, 50°C for 15 s, and 60°C for 4 min, ×35 cycles. DNA sequence was subsequently analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems).

**RESULTS**

We chose to use real-time quantitative MSP to accurately analyze clonal expansion of RASSF1A promoter methylation in various thyroid tissues. As shown in Table 1, based on a total allelic methylation of 25% as the cutoff point for each sample analyzed (a total of 65 samples), 0 of 14 (0%) normal thyroid tissues, 4 of 9 (44%) benign thyroid adenomas, 9 of 12 (75%) FTC tumors, and only 6 of 30 (20%) FTC tumors were identified as positive. The methylation level in 11 of 14 normal (tumor marginal) thyroid samples examined was zero, and three normal thyroid samples showed minimal allelic methylation (median ± SD = 0 ± 1.49%; Fig. 1). The 25% cutoff point was chosen here with a conservative assumption that all of the cells in a sample were tumor cells without normal cell contamination and without loss of heterozygosity in the RASSF1A locus. Thus, a cutoff point of 25% of total alleles for RASSF1A methylation implies that 25% of all of the tumor cells carry RASSF1A methylation if both alleles are methylated in one cell. In reality, microdissection of the tumor tissues is not 100% pure, and there may be also loss of heterozygosity in the RASSF1A locus. Therefore the 25% cutoff point of total alleles for RASSF1A methylation actually reflects a clonal expansion of >25% of the tumor cells. About 20% of PTC and 30% of FTC showed nearly 100% allelic methylation (Fig. 1), suggesting that in these tumors RASSF1A methylation occurred in both alleles (or in one allele with loss of heterozygosity in the locus carrying the second allele) in a cell with clonal expansion extending through the entire tumor.

We next sought to investigate the relationship between RASSF1A hypermethylation and the recently discovered BRAF mutation in thyroid neoplasms. Because the T1796A transversion mutation is the most common BRAF mutation in human cancers (25) and was found recently in PTC with a high frequency (2–7), we analyzed this mutation in the present study. Consistent with previous reports, we did not find BRAF mutation in any of the benign thyroid neoplasm and FTC samples examined in this study, including those that showed high level of RASSF1A methylation (data not shown). Interestingly, an
Table 2 Inverse correlation of RASSF1A methylation with BRAF mutation in papillary thyroid cancersa

<table>
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<tr>
<th>BRAF mutation status</th>
<th>RASSF1A methylation level</th>
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<tr>
<td></td>
<td>10% alleles</td>
</tr>
<tr>
<td>Negative</td>
<td>6/12 (50%)</td>
</tr>
<tr>
<td>Positive</td>
<td>2/18 (11%)</td>
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* Presented, respectively for BRAF mutation-negative and -positive papillary thyroid cancers, the number and percentage of cases that reached the indicated RASSF1A methylation level (percentage of the total alleles). The inverse relationship between BRAF mutation and RASSF1A methylation is statistically significant at both methylation levels shown (P = 0.0342 for the “10%” level and 0.0016 for the “>10–90%” level, per 2-tailed Fisher’s exact χ² test).

The inverse relationship between RASSF1A hypermethylation and BRAF mutation was observed in PTC; the PTCs that contained heavy RASSF1A methylation as shown in Fig. 1 were all negative for BRAF mutation, and none of the BRAF mutation-positive PTCs showed significant RASSF1A methylation. As can be seen in detail in Table 2, 6 of 12 (50%) of the BRAF mutation-negative PTCs showed RASSF1A hypermethylation at a level of >90% of the total alleles, whereas only 2 of 18 (11%) of the BRAF mutation-positive PTCs showed RASSF1A methylation. Moreover, these 2 latter tumors harbored only a small percentage (<10%) of methylated alleles. We similarly observed this inverse correlation between RASSF1A hypermethylation and BRAF mutation in thyroid tumor cell lines (Fig. 2). As shown in Fig. 2, heterozygous BRAF mutation occurred in thyroid tumor cell lines KAK-1, KAT-5, KAT-7, KAT-10, and ARO, but not in the C643 thyroid tumor cell line, whereas RASSF1A methylation occurred in 100% of the total alleles in the C643 cell line and only minimally in the other cell lines (ranging from 1% to 17% of the total alleles).

DISCUSSION

Our findings on RASSF1A hypermethylation in thyroid tumors are interesting for several reasons. First, it occurs at an early stage of thyroid tumorigenesis (i.e., in benign thyroid adenomas) and represents the first example of epigenetic inactivation of this tumor suppressor gene at a high prevalence in a common benign neoplasm. This contrasts with the genetic inactivation of the major tumor suppressor gene, p53, which generally occurs in late-stage undifferentiated thyroid cancers (26). Second, the prevalence of RASSF1A promoter methylation is similar in benign thyroid adenomas and FTC by conventional MSP (data not shown), and extensive clonal expansion of this epigenetic event is most common in these two types of thyroid tumors by real-time quantitative MSP (Fig. 1). Consistent with the histological similarities between benign adenomas and FTC, this RASSF1A methylation pattern supports the hypothesis that the latter originates from the former (1). Third, we observed for the first time that RASSF1A methylation occurred in an inverse correlation with BRAF mutation as shown by the data with PTC (Table 2) and thyroid tumor cell lines (Fig. 2). Our finding of a high prevalence and common clonal expansion of RASSF1A gene promoter methylation in benign adenomas, FTC, and the BRAF mutation-negative PTC thus provides the first epigenetic evidence suggesting that at least a portion of the latter two types of thyroid cancers may have been derived from benign thyroid adenoma.

Similar to our findings, a previous study found that PTC was associated with the lowest prevalence of RASSF1A hypermethylation among the thyroid cancers examined (20). This was presumably due to the possibility that, in that series of PTCs, a substantial portion of the samples were positive for BRAF mutation and, hence, negative for RASSF1A methylation. Although conventional MSP is a sensitive method to examine the prevalence of methylation, it is not a quantitative measure. Empirically, only a few methylated allele copies are often sufficient to generate an amplification band on MSP gels (data not shown). Conventional MSP used in the previous study (20) may thus have overestimated to some degree the prevalence of significant RASSF1A methylation in thyroid tumors (20).

On the basis of these data, we propose that epigenetic inactivation of the tumor suppressor gene RASSF1A, through aberrant promoter methylation, may play an important role in the formation of benign thyroid adenoma and its progression to FTC and BRAF mutation-negative PTC. Aberrant RASSF1A methylation may lead to progressive clonal expansion in the benign tumor and, when accumulation of such methylation reaches a critical level or, perhaps, when an additional genetic/epigenetic alteration occurs, may subsequently lead to

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Fig. 2. Inverse correlation of RASSF1A methylation with BRAF mutation in thyroid tumor cell lines. Shown for each cell line is the sequence TACATGCAAATCT in which nucleotide 1796 (as indicated by the arrow) is heterozygously mutated from T to A in cell lines KAK-1, KAT-5, KAT-7, KAT-10, and ARO, whereas the mutation is absent in the C643 cell line (wild-type allele). The extent of RASSF1A methylation in terms of percentage of the total alleles is indicated in the brackets for each cell line.
malignant transformation of benign adenomas to FTC and BRAF mutation-negative PTC. No benign adenoma examined by quantitative real-time MSP showed clonal expansion of RASSF1A methylation to more than half of the total alleles of the tumor sample (Fig. 1), suggesting that this level of RASSF1A methylation may represent a critical point where a benign thyroid neoplasm may undergo malignant transformation to cancer.

In their early cytogenetic and molecular genetic studies of thyroid tumors, Herrmann et al. (27) demonstrated a high frequency of loss of heterozygosity of all of the informative loci on chromosomal arm 3p in FTC and proposed that a tumor suppressor gene responsible for the progression from benign thyroid adenoma to FTC was located within this chromosomal arm. This tumor suppressor gene may be the RASSF1A gene, which is located at 3p21.3 (18). These earlier data are therefore consistent with the present idea that inactivation of the tumor suppressor gene RASSF1A indeed plays a role in thyroid tumorigenesis. Although many details of how RASSF1A inactivation by hypermethylation contributes to thyroid tumorigenesis are still unknown, its complete methylation in all of the cells observed in a subgroup of FTC and BRAF mutation-negative PTC (Fig. 1) suggests that the role of RASSF1A methylation may be a critical and indispensable one in the formation or progression of this subgroup of thyroid cancers.

In fact, the mutual exclusion between RASSF1A methylation and Ras mutations in other cancers (28, 29) and BRAF mutation in thyroid tumors (present study) suggests that RASSF1A methylation may play an independent and leading role in many of these tumors. Ras mutations and BRAF mutations are also mutually exclusive in various neoplasms, including colorectal carcinomas (30), colorectal polyps and adenomas (31), melanomas (32), and ovarian carcinomas (33), as well as thyroid cancers (4). Interestingly, another major thyroid tumor-related genetic alteration, RET/PTC rearrangement, was found recently to be mutually exclusive with BRAF mutation in thyroid cancers (2, 4). As illustrated in Table 3, for sporadic thyroid cancers in adult patients, in general, BRAF mutation occurred in ~45% of PTCs (ranging from 35% to 69%; Refs. 2–5); RET/PTC rearrangements occurred in about 20–35% of PTC and rarely in FTC (34), and Ras mutations occurred in about 15–20% of PTCs and 40–50% of FTCs (8, 35). Significant RASSF1A methylation (>50% of total alleles) occurred in 20% of PTCs and 60% of FTCs (Fig. 1). Therefore, RASSF1A hypermethylation completes a panel of genetic and epigenetic alterations of the Ras pathway components that apparently can each independently initiate or drive thyroid cancer formation and, together, are responsible for the formation or progression of almost all of the follicular epithelial cell-derived thyroid cancers.

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