Frequent Epigenetic Silencing of the CpG Island Promoter of RASSF1A in Thyroid Carcinoma

Undraga Schagdarsurengin, Oliver Gimn, Cuong Hoang-Vu, Henning Dralle, Gerd P. Pfeifer, and Reinhard Dammann

INTRODUCTION

Four types of thyroid carcinomas comprise >98% of all thyroid malignancies: (a) PTC; (b) FTC; (c) UTC; and (d) MTC (1). PTC may have a very benign course, whereas UTC belongs to the most aggressive human malignancies. PTC, FTC, and UTC originate from the thyroid follicular epithelial cells. In contrast, MTC derives from the parafollicular C cells and is the main type of hereditary thyroid cancer. Germ-line mutations of the proto-oncogene RET have been found in the majority of hereditary MTC (2). Somatic mutations of RET are involved in 30–50% of sporadic tumors (3). LOH of several chromosomal regions (1p, 3p, 3q, 10q, 11p, 13q, and 22q) has been reported in thyroid carcinomas (4). Particularly, LOH of the short arm of chromosome 3 is a common event in the pathogenesis of thyroid cancer and other solid tumors (5). Recently, we et al. (6, 7) have cloned and characterized the Ras association domain family 1A gene (RASSF1A) from the common homozygous deletion area at 3p21.3. RASSF1A is frequently inactivated in a variety of primary human cancers, including lung, breast, nasopharyngeal, and kidney carcinoma (6, 8–12). RASSF1A contains several interesting domains, including a Ras association domain, a diacylglycerol/phosphol ester binding domain, and a putative ATM kinase phosphorylation site (6, 13). The RASSF1C form was isolated by its binding to XPA in the yeast two hybrid system (6). However, the biological function of RASSF1 and its isoforms (A, B, C, D, E, F, and G) is still under investigation. The two main forms (RASSF1A and RASSF1C) are transcribed from distinct CpG island promoters (6). In lung cancer cell lines, which lack endogenous transcription, reinsertion of RASSF1A has been shown to reduce colony formation and anchorage-independent growth in soft agar (6, 10). Human cancer cells lacking RASSF1A transcription formed larger tumors compared with the same cells expressing RASSF1A in nude mice (6, 10). Thus, reinsertion of RASSF1A inhibits tumorigenicity in vitro and in vivo. Interestingly, mutational inactivation of this gene is very rare (<2%), and the main mechanism of its inactivation is through promoter methylation and LOH.

Here, we analyzed the epigenetic inactivation of RASSF1A and p16 INK4a in primary thyroid cancer (PTC, FTC, MTC, and UTC) and in thyroid cancer cell lines by promoter methylation analysis and transcriptional studies.

MATERIALS AND METHODS

Tissues and Cell Lines. A total of 43 thyroid tissues, including 1 PDTC, 5 MTC, 10 FTC, 9 UTC, 13 PTC, 1 goiter, and 4 normal thyroid tissues, were obtained from patients of the Department of Surgery of the University of Halle by surgical resection and were stored at −80°C until use. The local committee of medical ethics approved the use of human tissue, and all patients gave their consent. Each tumor was scored based on the TNM classification (14). Nine human thyroid cancer cell lines (8305C, 8505C, C643, FTC133, FTC236, FTC238, 1736, B-CPAP, and HTh74) were cultured in the recommended growth medium. Genomic DNA was extracted from frozen tissues and cultured cells by a standard phenol/chloroform procedure, and the total RNA was isolated by using TRIzol-Reagent (Life Technologies, Inc.).

Re-Expression of RASSF1A. Thyroid cancer cell lines were treated with 5-Aza-CdR (Sigma). Cells (2 × 10⁶) each were grown for 4 days in the presence of different concentrations of 5-Aza-CdR (0, 5, and 10 μM). RNA was isolated, and RT-PCR was performed as described below.

Bisulfit e Modification of the DNA and MSP Analysis. Methylation of the RASSF1A promoter region was determined by bisulfite modification of genomic DNA (6, 15). Promoter methylation was investigated by MSP as described by Herman et al. (16). For the methylation status of RASSF1A CpG

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3 The abbreviations used are: PTC, papillary thyroid carcinoma; MTC, medullary thyroid carcinoma; FTC, follicular thyroid carcinoma; UTC, undifferentiated thyroid carcinoma; TBE, Tri-borate EDTA; PTCD, poorly differentiated thyroid carcinoma; RT-PCR, reverse transcription-PCR; LOH, loss of heterozygosity; 5-Aza-CdR, 5-aza-2'-deoxycytidine; MSP, methylation-specific PCR; COBRA, combined bisulfite restriction analysis; TNM, Tumor-Node-Metastasis.
islands, the primer set RAM and RAU were used as described by Lo et al. (11). PCR was performed with methylation-specific primers RAM-1 (5’gtgttgaggtgttaatgtg) and RAM-2 (5’accccggaactaaaaaaga) and unmethylation-specific primers RAU-1 (5’tttggttggagtgtgttaatgtg) and RAU-2 (5’caaaccaccacaactaaacaaa) using 100 ng of the bisulfite-modified genomic DNA as templates for 36 (RAM-1/RAU-2) and 33 cycles (RAU-1/RAU-2) at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR products (10 μl) were analyzed on a 2% TBE agarose gel. To analyze the methylation status of the p16 gene, primers and conditions as described by Herman et al. (16) were used.

**RT-PCR Analysis.** RNA (1 μg) from thyroid cancer cell lines and tissues was reverse transcribed with primer LHE4 (5’ctgcagggggcgtgacct) located in exon 4 of RASSF1A (5’/H11032) and for RASSF1C: L27111 and U2G (5’gtgcttccgcctgacact). PCR products were separated on 2% TBE agarose gels and analyzed by densitometry.

**RESULTS**

**Hypermethylation of the RASSF1A Promoter Occurs in Thyroid Carcinomas.** In previous studies, we have shown that RASSF1A expression was missing in lung, breast, kidney, and ovarian cancer and that loss of transcription correlated with hypermethylation of the RASSF1A promoter. The only PDTC analyzed was FTC238, 8505C, C643, FTC133, FTC236, 1736, B-CPAP, 8305C, and HTh74. All thyroid cancer cell lines show complete methylation of the promoter CpG island by MSP (Fig. 1). Because the primers used for MSP are localized downstream of the transcription initiation site, we confirmed these results by COBRA (data not shown). The analyzed cell lines originated from different primary thyroid tumors: FTC (B-CPAP), UTC (8305C, 8505C, 1736, C643, and HTh74), and FTC metastasis (FTC133, FTC236, and FTC238). Therefore, we performed a detailed MSP analysis of the RASSF1A CpG islands in different primary thyroid carcinoma, including 1 PDTC, 5 MTC, 10 FTC, 9 UTC, and 13 PTC (Fig. 1 and Table 1). We could detect RASSF1A methylation in all types of thyroid carcinoma. The methylation frequency of the RASSF1A gene is 71% in the 38 thyroid carcinomas. The lowest methylation frequency was detected in FTC with 8 of 13 (62%) methylated cases. In 7 of 10 (70%) FTC, RASSF1A was inactivated. The only PDTC analyzed was FTC238. Therefore, we confirmed the MSP results by COBRA (data not shown). In Table 1, we summarize the methylation status of the RASSF1A gene depending on the TNM classification of thyroid carcinomas. RASSF1A inactivation was found in all tumor stages (T1 to T4) and in the invasive T4 cases (71%).

**Expression of RASSF1A in Thyroid Carcinomas.** We investigated the transcription of RASSF1A in thyroid cancer cell lines and primary tumors. In all nine cell lines, RASSF1A expression was missing (Fig. 2A and data not shown). We treated four thyroid carcinoma cell lines (1736, 8505C, C643, and B-CPAP) with different concentration of 5-Aza-dCR, a drug which blocks de novo methylation of the DNA after replication (17). After 4 days of treatment, we detected two different RASSF1 fragments by RT-PCR analysis, we detected two different RASSF1 fragments (Fig. 2B). The top 347-bp fragment corresponds to the full-length RASSF1A mRNA, and the bottom 240-bp fragment corresponds to the RASSF1B splicing variant (Fig. 2B). The F-form of RASSF1 skips exon 2αβ and encodes only the truncated protein kinase C1 domain (10). The highest level of RASSF1A transcript was detected in those samples, where the RASSF1A CpG island was unmethylated by MSP analysis:

![Fig. 1. MSP of the RASSF1A CpG island. The methylation status of the RASSF1A promoter region was analyzed by MSP in different primary thyroid tissues (PTC, MTC, FTC, and UTC), thyroid cancer cell lines (bottom panel: FTC236, 8505C, C643, FTC133, FTC236, 1736, B-CPAP, 8305C, and HTh74), and normal thyroid tissues (N8, N9, and N18). Methylation- (m) and unmethylation-specific (u) primers were used for MSP. The methylation-specific product (93 bp) and unmethylation-specific products (105 bp) were resolved on 2% TBE gel. HeLa-DNA and in vitro methylated DNA (Methyl) were used as controls for MSP.](image-url)

### Table 1 Summary of the methylation analysis

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<th>Case and TMN classification</th>
<th>RASSF1A methylation</th>
<th>p16 methylation</th>
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<tbody>
<tr>
<td>Thyroid cancer cell lines</td>
<td>9/9 (100%)</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>Primary thyroid carcinoma</td>
<td>27/38 (71%)</td>
<td>9/36 (25%)</td>
</tr>
<tr>
<td>T1</td>
<td>3/5 (60%)</td>
<td>1/5 (20%)</td>
</tr>
<tr>
<td>T2</td>
<td>4/6 (67%)</td>
<td>0/4 (0%)</td>
</tr>
<tr>
<td>T3</td>
<td>8/10 (80%)</td>
<td>3/10 (30%)</td>
</tr>
<tr>
<td>T4</td>
<td>12/17 (71%)</td>
<td>5/17 (29%)</td>
</tr>
<tr>
<td>N0</td>
<td>11/13 (85%)</td>
<td>1/12 (8%)</td>
</tr>
<tr>
<td>N1</td>
<td>14/23 (61%)</td>
<td>7/22 (32%)</td>
</tr>
<tr>
<td>Nm</td>
<td>2/2 (100%)</td>
<td>1/2 (50%)</td>
</tr>
<tr>
<td>Mo</td>
<td>12/21 (57%)</td>
<td>4/17 (21%)</td>
</tr>
<tr>
<td>M1</td>
<td>6/8 (75%)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>Mx</td>
<td>99/100 (%)</td>
<td>59/56 (%)</td>
</tr>
<tr>
<td>FTC</td>
<td>7/10 (70%)</td>
<td>1/10 (10%)</td>
</tr>
<tr>
<td>UTC</td>
<td>7/9 (78%)</td>
<td>59/56 (%)</td>
</tr>
<tr>
<td>MTC</td>
<td>4/5 (80%)</td>
<td>0/4 (0%)</td>
</tr>
<tr>
<td>PDTC</td>
<td>1/1</td>
<td>0/1</td>
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<tr>
<td>Goiter tissue</td>
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<td>0/1</td>
</tr>
<tr>
<td>Normal thyroid tissues</td>
<td>1/4 (25%)</td>
<td>1/4 (25%)</td>
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* N0 and M0, no metastasis detected; N1 and M1, metastasis detected; Nm and Mx, status of metastasis not available.
Carcinomas and in four of eight (50%) cell lines (Table 1). No
p16
3). In summary, methylation was detected in four MTC and a PDTC. Given that
presence of bisulfite-modified DNA (Fig. 1). In addition, we investi-
RASSF1
in this cell line, because DNA amplification of
p16
inactivation of
p16
Methylation of
p16
in thyroid cancer. Transcripts were
amplified by RT-PCR with primers from exon 1β and exon 4. The RASSF1A fragment was 347 bp, and the RASSF1F fragment was 240-bp long. The methylation status of the RASSF1A CpG island is indicated as methylated (m) and unmethylated (u). Expression of GAPD was determined as a control for RNA integrity.

Fig. 2. Expression of RASSF1 in thyroid carcinomas. A, re-expression of RASSF1 by treatment with 5-Aza-Cdr in four thyroid cancer cell lines (1736, 8505C, C643, and B-CPAP). The cell lines were treated for 4 days with the indicated concentra-
tions of 5-Aza-Cdr. RASSF1A and RASSF1F were analyzed by isoform-specific RT-PCR. Ex-
pression of GAPD was determined as a control for RNA integrity. B, expression analysis of RASSF1A and RASSF1F in thyroid cancer. Transcripts were
indicated as methylated (m) and unmethylated (u). Expression of GAPD was determined as a control for RNA integrity.

N18, HeLa, UTC16, PTC51, MTC13, PTC28, and FTC14 (Fig. 2B). Loss of RASSF1A transcription correlated with hypermethylation of the RASSF1A CpG island.

Methylation of
p16
in Thyroid Cancer. Epigenetic silencing of the cyclin-dependent kinase inhibitor
p16
is a common and frequent event in solid tumors (18). Therefore, we examined the inactivation of
p16
in thyroid carcinoma by MSP (16). Methylation of
p16
was analyzed in nine thyroid cancer cell lines (Fig. 3). In four cell lines (8305C, 8505C, 1736, and B-CPAP), the
p16
CpG island was hypermethylated, and in four others (FTC133, FTC236, FTC238, and C643), methylation was undetectable (Fig. 3). In the HTh74 cell line, we could not obtain products with both
p16
MSP primer pairs in several DNA isolations (data not shown). Most likely, 
p16
is deleted in this cell line, because DNA amplification of RASSF1 indicated the presence of bisulfite-modified DNA (Fig. 1). In addition, we inves-
tigated the methylation status of
p16
in 36 primary thyroid tumors (Fig. 3). In summary, 
p16
was hypermethylated in 9 of 36 (25%) primary carcinomas and in four of eight (50%) cell lines (Table 1). No
p16
methylation was detected in four MTC and a PDTC. Given that
p16
mutations are most common in hereditary melanoma, we analyzed the
p16
gene for mutations in the MTC, thought to be the hereditary form of thyroid cancer. However, no
p16
mutation was detected in the 5 MTCs. Hypermethylation of 
p16
was found in 10 and 25% of FTC and PTC, respectively. The highest frequency of
p16
inactivation was found in the aggressive UTC (56%).
p16
methylation was detected in one normal sample (Fig. 3), which also exhibited aberrant RASSF1A methylation (N19). In Table 1, we summarize the methylation status of
p16
depending on the TNM classification.

p16
inactivation was detected in most tumor stages and in the invasive
T4 cases (29%). In 26% of the tumors, neither
p16
nor RASSF1A were inactivated, and in 22% of carcinomas, both genes were silenced. Interestingly, 90% of tumors with
p16
inactivation had also RASSF1A inactivation. However, RASSF1A inactivation was almost three times more frequent in thyroid carcinoma compared with
p16
hypermethylation.

DISCUSSION

We et al. (6, 8–12) have reported frequent inactivation of RASSF1A in a variety of primary human cancers, including lung, breast, naso-
ephrangyeal, kidney, and ovarian carcinoma. In this study, we demon-
strated that RASSF1A silencing is a common event in thyroid carcinomas. Primary thyroid carcinomas (71%) and 100% of thyroid cancer cell lines showed RASSF1A inactivation. The highest methy-
lation (80%) was found in the more aggressive forms (UTC and MTC) of thyroid carcinoma, whereas in the more benign PTC, RASSF1A hypermethylation (62%) was less pronounced. Therefore, RASSF1A inactivation correlated with the malignancy of the primary tumors. Interestingly, RASSF1A inactivation was higher in tumors, which developed distant metastasis. In other studies, a similarly high fre-
quency of RASSF1A inactivation has been observed in 62% of breast carcinomas (8), 56% of renal cell carcinomas (12), and 67% of nasopharyngeal carcinomas (11). RASSF1A inactivation has been found in ~30–40% of non-small cell lung cancers and ovarian tumors (6, 9, 10, 12). In colon cancer and cervical cancer, RASSF1A is less frequently inactivated (9, 12). Both alleles of a tumor suppressor gene need to be inactivated in the classical and revised two-hit hypothesis (19). LOH and epigenetic inactivation of the RASSF1A gene are very frequent events. Mutations of the coding sequence of the RASSF1 gene are rare. RASSF1A inactivation of both alleles is a critical event in small cell lung carcinogenesis (9, 10). In other types of cancer, RASSF1A promoter methylation together with LOH have been found less frequently (9). RASSF1A may belong to the class of haplo-
sufficient tumor suppressor genes that promotes tumor formation through the inactivation of only one allele. A second possibility is that another genetic lesion is present to alter the pathway in which
RASSF1A inhibits tumorigenesis, e.g., oncogenic Ras may play the counterpart for loss of RASSF1A function in carcinogenesis. Vos et al. (20) have shown that RASSF1C binds Ras, and overexpression of RASSF1C induces apoptosis. However, our own data indicate that the pro-apoptotic effect may require heterodimerization with the novel Ras effector NORE1, and RASSF1 binds to Ras only very weakly by itself (21). In addition to the Ras association domain and the protein kinase C1 domain, which may be involved in signal transduction and growth promoting, RASSF1A also contains an in vitro phosphorylation site for ATM kinase (13), and RASSF1C was isolated by its binding with the DNA repair protein XPA (6). However, which of these possible functional pathways may inhibit tumorigenesis remains to be worked out.

Inactivation of p16INK4a is a common and early event in the development of cancer. The highest frequency of p16 inactivation (30–40%) has been reported in tumors of the colon, lung, brain, esophagus, stomach, pancreas, and in 48% of lymphomas (18). Here, we detected methylation of p16 in 25% of primary thyroid carcinomas and inactivation in 56% of thyroid cell lines (19). The frequency of p16 inactivation in PTC (25%) is consistent with 30% of silencing in a similar study (22). The highest frequency of p16 inactivation (56%) was found in UTC. Thus, a high frequency of p16 and RASSF1A inactivation in UTC may be responsible for the malignity of this carcinoma, which belongs to the most aggressive human cancer. In 26% of tumors, neither p16 nor RASSF1A were inactivated. Elisei et al. (22) have reported p16 silencing in 25% of follicular adenomas. Follicular adenomas are benign tumors of the thyroid, which often occur in goiter. It is not known whether FTC develops through preexisting follicular thyroid adenoma or not. Therefore, it will be interesting to analyze RASSF1A inactivation in follicular adenoma and other benign changes of the thyroid gland. Thus, inactivation of RASSF1A may be an early event in the pathogenesis of thyroid cancer and could be used as a marker for carcinogenesis.

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