

Inactivation of *RASSF1A* tumor suppressor gene by aberrant promoter hypermethylation in human pituitary adenomas

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Aberrant promoter methylation and resultant silencing of several tumor suppressor genes play an important role in the pathogenesis of many tumor types. The human *Ras association domain family 1A* gene (*RASSF1A*), recently cloned from the lung tumor locus at 3p21.3, was shown to be frequently inactivated by hypermethylation of its promoter region in a number of malignancies. We have investigated the expression and epigenetic changes of this novel universal tumor suppressor gene in pituitary adenomas and correlated the data with clinicopathologic findings. Fresh frozen normal pituitary tissues and 52 primary pituitary adenomas including all major types were examined. Methylation-specific polymerase chain reaction (MSP), combined bisulfite restriction analysis (COBRA), bisulfite sequencing and semiquantitative reverse transcription-polymerase chain reaction were used to analyze DNA promoter methylation status and the mRNA expression of *RASSF1A*, respectively. High levels of *RASSF1A* transcript and no methylation of the *RASSF1A* promoter were found in normal pituitary tissues. *RASSF1A* promoter methylation was detected in 20 of 52 (38%) adenomas including all major types of pituitary adenomas. However, a lower frequency of methylation of the *RASSF1A* promoter was found in gonadotroph cell adenomas (15%) compared with growth hormone cell, prolactin cell, or adrenocorticotrophic hormone cell adenomas (54, 46 and 50%, respectively). Methylation frequency was higher in the most aggressive adenomas (87% in grade IV tumors, $P=0.0163$). In addition, methylation of the *RASSF1A* promoter potentially correlated with higher labeling index of the proliferation marker Ki-67 ($P=0.1475$). Loss or significant reduction of *RASSF1A* messenger RNA transcripts was identified in 18 of 20 (90%) adenomas with hypermethylation of *RASSF1A* ($P<0.0001$). Our data suggest that promoter hypermethylation of *RASSF1A* and resultant alterations of *RASSF1A* expression may play a critical role in pituitary tumorigenesis and may be involved in tumor progression.

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Pituitary adenomas are common and potentially serious neoplasms. They can cause mood disorders, sexual dysfunction, infertility, obesity, visual disturbances, hypertension, diabetes mellitus and

accelerated heart disease. Although the majority of tumors are benign, a proportion may invade the surrounding structures such as the sphenoid sinus, the cavernous sinus, and even the brain. The pathogenetic mechanisms underlying pituitary adenoma formation and progression remain unclear. The genetic mutations in classic oncogenes and tumor suppressor genes are rarely found in these tumors.¹ Progress in understanding the molecular basis of pituitary adenomas might allow the development of early diagnostic markers and novel therapeutic strategies.

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Aberrant methylation of normally unmethylated CpG islands in promoter regions of genes represents an epigenetic change that results in transcriptional inactivation of tumor suppressor genes (TSGs). While genetic events such as mutation, deletion, or rearrangement are well known, aberrant methylation is recognized as an additional mechanism contributing to tumorigenesis.^{2,3}

The methylation of CpG islands in several TSGs has been found in virtually every type of human neoplasms²⁻⁴ including pituitary tumors.⁵ The mechanism of p16 downregulation probably involves cyclin-dependent kinase inhibitor 2A (*CDKN2A*) methylation in most types of pituitary adenoma, but remain to be determined in growth hormone (GH) cell adenomas;^{6,7} and *retinoblastoma 1 (RB1)* gene methylation with tumor subtype specificity was described in pituitary tumors.⁸ Loss of E-cadherin associated with methylation of *cadherin 1 (CDH1)* has been identified in sparsely granulated GH-cell adenomas,⁹ but not in prolactinomas.¹⁰ Preferential loss of death-associated protein kinase (DAPK) expression in invasive pituitary tumors was reported to be associated with CpG island methylation.¹¹ Thus, inactivation of TSGs due to hypermethylation of CpG islands may play an important role in pituitary tumorigenesis and needs further investigation.

Recently, the *Ras association domain family 1 (RASSF1)* gene was found as a tumor suppressor gene at 3p21.3 by its frequent epigenetic silencing and loss of heterozygosity (LOH) in lung cancers and lung cancer cell lines.¹² *RASSF1* encodes more than seven isoforms including two major transcripts, known as A and C, which are derived from alternative mRNA splicing and use of different promoter sites. Hypermethylation of the *RASSF1A* promoter has been frequently identified in breast, ovarian, nasopharyngeal, kidney, bladder, gastric, prostate, thyroid, esophageal squamous cell carcinomas, uterine cervical adenocarcinoma and malignant cutaneous melanoma.¹³⁻²³ These findings suggested that *RASSF1A* may be a universal TSG involving in tumorigenesis.

Furthermore, the ability of *RASSF1A* to suppress the malignant phenotype has been confirmed both *in vitro* and *in vivo*. Re-expression of *RASSF1A* gene in lung and renal cell carcinoma cell lines lacking endogenous expression of *RASSF1A* suppressed colony formation, anchorage-independent growth,^{12,16,24} and tumor formation in nude mice.^{12,24} In addition, RASSF1C protein was found to bind Ras in a GTP-dependent manner both *in vitro* and *in vivo* and mediate the apoptotic effects of oncogenic Ras.²⁵ However, the putative tumor suppressive function of RASSF1C requires much further investigation.

To understand whether the promoter hypermethylation of *RASSF1A* could play a role in human pituitary tumorigenesis, we examined the frequency of methylation of *RASSF1A* gene promoter in

pituitary adenomas of various types; the expression of the *RASSF1A* gene was also investigated. In addition, we analyzed the potential clinicopathological implication of hypermethylation of *RASSF1A* in pituitary adenomas.

Materials and methods

Human Pituitary Tissues and Adenomas

Four normal human adenohypophyses were obtained at autopsy from patients with no evidence of endocrine abnormality; they were examined histologically and using immunocytochemistry to exclude the possibility of incidental pathology. A total of 52 pituitary adenoma specimens were obtained at the time of surgery at Tokushima University Hospital (Tokushima, Japan) and Toranomon Hospital (Tokyo, Japan). All samples were frozen and stored at -70°C . Tumors were characterized based on the clinical, radiological, histological, and immunohistochemical features (Table 1).²⁶ There were 31 clinically functional tumors (13 somatotroph adenomas, one mammosomatotroph adenoma, 11 lactotroph adenomas, four corticotroph adenomas associated with Cushing's disease, two thyrotroph adenomas) and 21 clinically nonfunctioning adenomas (four silent corticotroph adenomas, 13 gonadotroph adenomas, three silent subtype three adenomas, and one null cell adenomas characterized by lack of immunoreactivity for all anterior pituitary hormones). Tumor size and invasiveness were defined on the basis of preoperative radiological investigations and operative findings and with a modified Hardy's classification.²⁷ Grade I tumors are microadenomas (<1 cm in diameter) and grade II tumors consisted of enclosed macroadenomas (≥ 1 cm in diameter) with or without suprasellar extension. Both grade I and II tumors are defined as noninvasive. Grade III tumors show local invasiveness with evidence of bony destruction and tumor within the sphenoid and/or cavernous sinus. Grade IV tumors demonstrate CNS/extracranial spread with or without metastases. Grade III and IV tumors were considered to be invasive. Thus, 52 tumors included five tumors of grade I, 24 tumors of grade II, 17 tumors of grade III, and six tumors of grade IV (29 noninvasive and 23 invasive adenomas; Table 1).

DNA Isolation and Sodium Bisulfite Modification

DNA was extracted using the Qiagen DNeasy Tissue Kit (Qiagen, Stanford, CA, USA) according to the manufacturer's protocol. Genomic DNA was modified by sodium bisulfite treatment and purified using the CpGenome DNA Modification Kit (Intergen, Purchase, NY, USA) according to the manufacturer's recommendations.

Table 1 Summary of *RASSF1A* methylation in normal pituitary tissues and pituitary adenomas

Variable	Case numbers	RASSF1A methylation		P
		+	-	
Normal Patients	4	0	4	
Age (years)	52	20 (38%)	32 (62%)	0.469
		49.2±4.1	45.1±2.5	
Gender				0.0391
Male	25	6 (24%)	19 (76%)	
Female	27	14 (52%)	11 (48%)	
Functional				0.2276 ^a
GH	13	7 (54%)	6 (46%)	
GH/PRL	1	0	1 (100%)	
PRL	11	5 (46%)	6 (54%)	
ACTH	4	2 (50%)	2 (50%)	
TSH	2	0	2 (100%)	
Total	31	14 (45%)	17 (55%)	
Non-functional				
ACTHs	4	2 (50%)	2 (50%)	
FSH/LH	13	2 (15%)	11 (85%)	
Subtype 3	3	2 (67%)	1 (33%)	
Null cell	1	0	1 (100%)	
Total	21	6 (29%)	15 (71%)	
Grade				0.0163 ^b
I	5	2 (40%)	3 (60%)	
II	24	8 (33%)	16 (67%)	
III	17	5 (29%)	12 (71%)	
IV	6	5 (83%)	1 (17%)	
Invasion				0.5078
Invasive	23	10 (44%)	13 (56%)	
Noninvasive	29	10 (35%)	19 (65%)	
Ki-67 LI (%)				0.1475
≥2.5	12	7 (35%)	5 (16%)	0.1067
<2.5	40	13 (65%)	27 (84%)	

Normal, normal pituitary tissue; GH, somatotroph adenoma; GH/PRL, mammosomatotroph adenoma; PRL, lactotroph adenoma; ACTH, corticotroph adenoma; ACTHs, silent corticotroph adenoma; TSH, thyrotroph adenoma; FSH/LH, gonadotroph adenoma; subtype 3, silent subtype 3 adenoma; null cell, null cell adenoma; LI, labeling index.

^aFunctional vs nonfunctional.

^bI+II+III vs IV.

Methylation-Specific PCR

The promoter methylation status of *RASSF1A* was investigated by methylation-specific PCR (MSP) assay as described previously.^{24,28} Two sets of *RASSF1A* promoter-specific primers described by Burbee *et al*²⁴ were used to specifically amplify methylated and unmethylated DNA sequences. CpGenome™ Universal Methylated DNA (Intergen) was used as methylated control and bisulfite-modified genomic DNA from HeLa cells served as an unmethylated control.^{12,20} Polymerase chain reaction (PCR) products were separated in 2%

agarose gels and visualized under ultraviolet (UV) illumination. The bisulfite reaction and MSP for all samples were repeated to confirm methylation status. Both methylated and unmethylated PCR products were purified for directly sequencing using the NucleoSpin® Extract Kit (Macherey-nagel, Düren, Germany). Cycle sequencing was performed using the BigDye Terminator V1.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Combined Bisulfite Restriction Analysis (COBRA) and Bisulfite Sequencing

The methylation status of the *RASSF1A* promoter region was also determined by COBRA²⁹ and bisulfite genomic sequencing³⁰ with primers described by Dammann *et al*.^{12,13} An amount of 100 ng of bisulfite-treated DNA was amplified with primers MU379 and ML730 in 20 μl of reaction buffer containing 200 μM of each deoxynucleotide triphosphate and *Taq* polymerase (AmpliTaq Gold; PE Applied Biosystems) and initial incubation at 95°C for 10 min, followed by denaturation at 95°C for 15 s, 55°C for 15 s, and 74°C for 30 s for 20 cycles. A seminested PCR was performed using 1 μl of the initially amplified products, and an internal primer ML561 and the primer MU379 in 50 μl of total reaction volume with similar conditions as described for the preceding PCR amplification but for 30 cycles. The PCR products were purified using QIAquick PCR purification kits (Qiagen, Valencia, CA, USA).

For COBRA, 300 ng of the PCR products were digested with 10 U of *TaqI* (New England Biolab, Beverly, MA, USA) according to the conditions specified by the manufacturer of the enzyme and analyzed on a nondenaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination. CpGenome™ Universal Methylated DNA and DNA from HeLa cells served as positive control and negative control respectively. *TaqI* digestion of the 205-bp PCR product generates three fragments of 93, 81 and 31 bp on complete digestion or partially digested fragments of 174 and 112 bp. The density of resultant DNA bands on a gel was estimated by densitometric scanning of ethidium bromide-stained gels and analyzed with computer software (Image, NIH, Bethesda, MD, USA). For each case, the proportion of *TaqI* resistant (unmethylated) was calculated relative to the PCR product generated in the same reaction that was not subject to restriction digest.

PCR products were sequenced directly to obtain average methylation levels. Further, the PCR products were ligated into the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions, where six clones were sequenced. All of the described sequences were determined by

cycle sequencing and run on an automated DNA sequencer to determine the methylation status.

For comparison, we analyzed the CpG islands in promoter of *RASSF1C* by COBRA and bisulfite genomic sequencing as described previously.³¹ PCR reactions similar to those described above were performed. For the first PCR reaction, primers M1305 and M1627 were used. For the nested PCR, the internal primers M1318 and M1599 were used.³¹ The analyzed 311-bp promoter fragment contains 38 CpGs, one Sp1 consensus binding site and the putative transcription and translation initiation sites of *RASSF1C*. This methylated fragment has five *Bst*UI sites and digestion results in bands of 140, 89, 31, 21, 16 and 14 bp. For COBRA, 300 ng of the PCR products were digested with 10 U of *Bst*UI (New England Biolab) and analyzed on a nondenaturing 6% polyacrylamide gel. Furthermore, PCR products were subjected for directly sequencing.

Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNA was extracted using ISOGEN (NIPPON GENE, Toyama, Japan) according to the manufacturer's protocol. Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed as described.^{17,18} The primers for RT-PCR were described by Dammann *et al.*¹² Briefly, RNA (0.2 μ g) was preassociated with 50 pmoles of an antisense primer in exon 4. After the RT reaction with StrataScript RT (Stratagene, La Jolla, CA, USA), diluted cDNA (12.5 ng/50 μ l PCR reaction) was used as template for PCR; 35 cycles were determined to be within the logarithmic phase of amplification and yielded reproducible results with sense primer in exon 2 $\alpha\beta$ for *RASSF1A* or in exon 2 γ for *RASSF1C*. RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as an internal control using primers described previously.²⁴ RT-PCR products (10 μ l) were resolved on 2% agarose gels. Quantitation of *RASSF1A* and *C* expression levels was achieved by densitometric scanning of ethidium bromide-stained gels. The levels of expression of *RASSF1A* and *C* were analyzed with computer software (Image, NIH) and shown as the ratio of *RASSF1A* mRNA to *GAPDH* mRNA or the ratio of *RASSF1C* mRNA to *GAPDH* mRNA.

Immunohistochemical Analysis of Ki-67

Ki-67 antigen immunolocalization based on the labeled streptavidin-biotin method was performed on sections from representative blocks of paraffin-embedded tissues used for pathology diagnosis. Ki-67 mouse monoclonal antibody (clone MIB-1, 1:500 dilution; Immunotech, Marseilles, France) was used as in our previous study.¹⁰ The Ki-67 labeling index (LI) was determined by counting the number of

positive cells in a total of 1000 tumor cells observed in several representative high-power fields ($\times 400$).

Statistical Analyses

Using StatView J-4.5 software, Mann-Whitney *U*-test, χ^2 -test and Spearman's correlation coefficient by rank were performed to determine the significance of associations between different variables. The level of statistical significance was $P < 0.05$.

Results

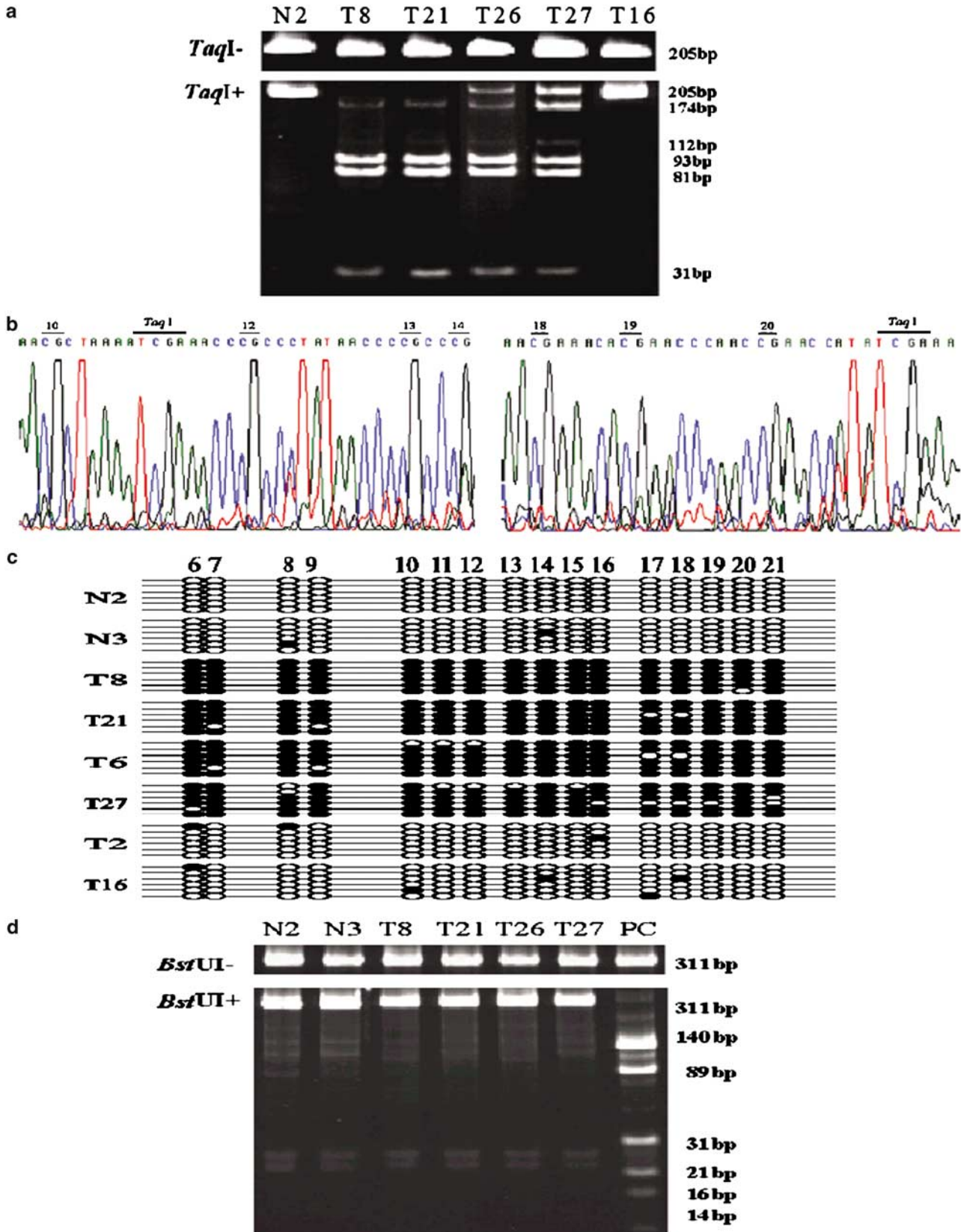
Methylation Status of *RASSF1A* in Pituitary Tumors

At first we used MSP to investigate the epigenetic changes of *RASSF1A*. We analyzed promoter methylation in four normal pituitary tissues and 52 pituitary adenomas. The methylation region includes 16 CpG sites that were previously found by MSP to be associated with reduced *RASSF1A* expression²⁴ (Figure 1a). Representative results are shown in Figure 1b–d. We then analyzed the relationship between *RASSF1A* methylation status and the clinicopathological characteristics of 52 patients. The results are summarized in Table 1. Hypermethylation of the promoter region of *RASSF1A* was detected in 20 of 52 (38%) pituitary adenomas, but not in four normal pituitary tissues, suggesting that *RASSF1A* promoter hypermethylation is tumor-specific in the pituitary. Unmethylated bands in MSP were detected in 45 of 52 (87%) adenoma samples and in all four normal tissues. In summary, among 52 pituitary adenomas, seven cases had only methylated alleles, 13 cases had both methylated and unmethylated alleles, and the other 32 cases had only unmethylated alleles as did four normal tissues (Figure 1b, c). Methylated patterns in *RASSF1A* were found in all major types of pituitary adenomas including seven of 13 (54%) somatotroph adenomas, five of 11 (46%) lactotroph adenomas, two of four (50%) functioning corticotroph adenomas, two of four (50%) silent corticotroph adenomas, two of 13 (15%) gonadotroph adenomas, and two of three (67%) silent subtype 3 adenomas. The *RASSF1A* promoter was not methylated in one mammosomatotroph adenoma, two thyrotroph adenomas, and one null cell adenoma. The difference in frequency between functional tumors (14 of 31, 45%) and nonfunctional tumors (six of 21, 29%) was not statistically significant. However, the frequency of *RASSF1A* methylation in gonadotroph adenomas is noticeably lower than in the other pituitary tumor types.

RASSF1A methylation was detected more in grade IV tumors (83%) than in grade I, II, III tumors (40, 33, 29%; respectively) ($P = 0.0163$). In addition, the frequency of *RASSF1A* methylation was higher in invasive tumors (44%) than in noninvasive tumors (35%), although this trend did not reach

than in *RASSF1A* unmethylated adenomas (1.15 ± 0.2), and higher Ki-67 LI ($\geq 2.5\%$) were observed more frequently in *RASSF1A* methylated

adenomas, although these trends did not reach statistical significance ($P=0.1475$ and 0.1067 , respectively).



In addition, the specificity of MSP was confirmed by direct sequencing. Six unmethylated MSP products from four normal tissues and two tumors, and 10 methylated MSP products from 10 tumors were directly sequenced. In unmethylated MSP products, all cytosine nucleotides including those in the CpG islands changed to thymines as a result of bisulfite modification. However, in methylated MSP products, cytosine nucleotides in the CpG islands remained cytosine (Figure 1d).

Analysis of Methylation Status of *RASSF1A* and *RASSF1C* by COBRA and Bisulfite Sequencing

Since MSP yielded both methylated and unmethylated bands in 13 samples, we further used COBRA and bisulfite sequencing to investigate the methylation status of *RASSF1A* in four normal tissues and 17 tumors. These 16 CpG sites examined by COBRA and bisulfite sequencing contained CpG sites from six to 21 in Figure 1. We obtained consistent results by COBRA with *TaqI* digestion (Figure 2a), bisulfite sequencing (Figure 2b, c) and MSP. COBRA showed positive results in 10 tumors. Four of 10 tumors showed total digestion suggesting complete CpG sites methylation, those tumors with only methylated bands in MSP. Six other tumors that had both methylated and unmethylated alleles by MSP showed a low proportion (3–18%) of undigested bands, in addition to completely digested bands. Bisulfite direct sequencing also detected a complete or nearly complete CpG methylation pattern in these 10 tumors. PCR products from four normal pituitary tissues and seven tumors, defined as unmethylated cases by MSP, were not digested by *TaqI* and also were shown to be unmethylated in all 16 CpG sites by bisulfite direct sequencing. The results of bisulfite direct sequencing were further confirmed by analyzing subclones of PCR products from two normal tissues and six tumors (Figure 2c).

Thus despite a little difference in DNA promoter regions examined and endogenous limitations of each method, the results from these three methods were consistent in this small group.

To prove the specificity of our data showing promoter methylation of *RASSF1A*, we analyzed the methylation status of the promoter of *RASSF1C* by COBRA and direct sequencing. Four normal pituitary tissues and 52 pituitary adenomas were unmethylated at all 38 CpG sites of *RASSF1C* (Figure 2d). These results prove that the methylation of CpG islands in *RASSF1A* promoter is a gene-specific event in pituitary adenomas.

Expression of *RASSF1A* and *C*

Next we analyzed expression of the *RASSF1A* gene in 52 pituitary adenomas. For comparison, *RASSF1C* gene expression was also analyzed. All four normal pituitary tissues as well as HeLa cells

expressed easily detectable levels of *RASSF1A* (*RASSF1A*/*GAPDH*, 0.71–0.80, mean 0.75) (Figure 3). Unfortunately, we were not able to obtain matched samples of normal pituitary tissue for each tumor; however, based on this consistent observation, we arbitrarily classified expression levels of

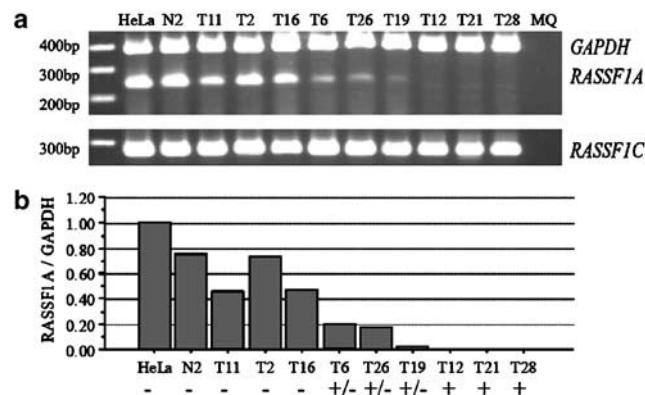


Figure 3 *RASSF1A* and *C* expression levels in normal pituitary tissues and pituitary tumors. (a) *RASSF1A* (242 bp) and *RASSF1C* (272 bp) transcripts were analyzed by semiquantitative RT-PCR using isoform-specific primers. *GAPDH* expressions were used as endogenous control for RNA integrity and as templates for quantitative analysis. N, normal tissues; T, tumor tissues; MQ, water as a template. (b) Ratios of *RASSF1A* mRNA to *GAPDH* mRNA in normal and tumor samples. The methylation status of samples was indicated. +, only methylated alleles; -, only unmethylated alleles; +/-, both methylated and unmethylated alleles.

Table 2 Correlation of *RASSF1A* methylation and expression of *RASSF1A* in pituitary adenomas

RASSF1A expression	Case numbers	RASSF1A methylation		P
		+	-	
-	11	11 (100%)	0	<0.0001
± ^a	11	7 (64%)	4 (36%)	
+	30	2 (7%)	28 (93%)	

^a±: significantly lower expression of *RASSF1A*.

less than one-half of this value, that is, *RASSF1A*/*GAPDH*, <0.38 as significant reduction (Figure 3). Loss or significant reduction of *RASSF1A* expression was found in 42.3% (22/52) of pituitary adenomas (Table 2 and Figure 3). These results demonstrate that loss or significant reduction of *RASSF1A* expression was frequent in pituitary adenomas.

RASSF1C transcripts were easily detectable in normal pituitary tissues and all pituitary adenomas. None of the tumors exhibited a significantly reduction or overexpression of *RASSF1C* (Figure 3a), proving that the differential promoter methylation of *RASSF1A* and *RASSF1C* is functionally important in pituitary tumors.

Correlation between RASSF1A Promoter Hypermethylation and Loss or Reduction of RASSF1A Expression

Four normal pituitary tissues with unmethylated alleles showed high levels of *RASSF1A* mRNA, comparable to that of HeLa cells. In 28 of 32 unmethylated tumors, *RASSF1A* mRNA levels were within the normal range. In four unmethylated adenomas, mRNA levels were low. In contrast, *RASSF1A* mRNA was not detected in 11 tumors with methylated *RASSF1A* promoters; seven other methylated tumors showed a significant reduction of *RASSF1A* expression. Just two methylated adenomas showed lower but not significantly reduced mRNA expression. Overall, there was a significant correlation between hypermethylation of *RASSF1A* promoter and abnormal expression of *RASSF1A* ($P < 0.0001$, Table 2, Figure 3b).

Discussion

Recently, epigenetic changes in cancer have gained considerable attention as a mechanism of silencing TSGs.³² Several major cancers such as lung, breast, colon, and prostate carcinomas have been widely studied for promoter hypermethylation of TSGs.^{4,33} Methylation of the TSGs *CDKN2A*, *RB1*, *CDH1* and *DAPK* has been identified in pituitary tumors.^{6–8,10}

Hypermethylation of CpG islands in the *RASSF1A* promoter and its associated gene silencing have been reported in a variety of tumors including endocrine tumors such as small cell lung cancer, thyroid carcinoma and pancreatic endocrine tumors.^{12,20,34} In the current study, we demonstrate *RASSF1A* promoter hypermethylation in 38% of pituitary adenomas including all the major clinically functioning and hormonally inactive types. *RASSF1A* methylation was significantly associated with loss or reduction of mRNA expression in these pituitary adenomas. Noticeable, *RASSF1A* methylation was detected in 11 tumors which exhibited no *RASSF1A* expression and in other seven tumors which showed significant reduction of *RASSF1A* expression. In contrast, the *RASSF1A* promoter was unmethylated and mRNA levels were high in normal pituitary tissues. As, there is no working antibody, we could not further analyze the relationship between methylation status and protein expression of *RASSF1A*. Nevertheless, our findings indicate that epigenetic inactivation of *RASSF1A* is associated with pituitary tumorigenesis.

There was no significant correlation between *RASSF1A* methylation and patient age, thus methylation of *RASSF1A* in pituitary adenomas is not attributable to the aging process, as has been described for other genes.³⁵ Although methylated *RASSF1A* in tumors was more frequently detected in women than in men, the reason for this gender-dependent difference is unclear and requires confirmation in larger numbers of cases. The frequency

of *RASSF1A* methylation was not significantly different between functional and nonfunctional groups. However, it is interesting to note that gonadotroph adenomas show a lower frequency of *RASSF1A* methylation (15%) compared to the other three major types: somatotroph, lactotroph and corticotroph adenomas (54, 46 and 50%; respectively). This tumor type-specific alteration, which is similar to that reported for *CDKN2A*, *RB1* and *CDH1*, indicates that different types of pituitary adenomas may have distinct etiologic factors and pathogenetic mechanisms.

Previous studies attested to an association of *RASSF1A* methylation with tumor aggressiveness, poor prognosis or tumor stage in several tumor types including bladder cancer, esophageal squamous cell carcinoma, and stage I lung adenocarcinoma.^{21,36,37} We demonstrate here that *RASSF1A* methylation is detected in all grades of pituitary adenoma and lacks a strict correlation with tumor invasiveness; nevertheless, hypermethylation of *RASSF1A* occurred more frequently in grade IV adenomas (83%) that exhibit a more aggressive behavior. These findings suggest that in addition to a role in tumorigenesis, *RASSF1A* methylation may also regulate the progression of pituitary adenoma. However, the numbers of grade I and IV tumors in this series is small, and further investigation of larger series is required.

The precise mechanism of the tumor-suppressing activity of *RASSF1A* is still unclear, and more biochemical and genetic data are needed to understand its role in tumorigenesis. Recently, Shivakumar *et al*³⁸ reported that *RASSF1A* can inhibit cell proliferation and block cell cycle progression by preventing cyclin D1 accumulation. Furthermore, Song *et al*³⁹ reported that *RASSF1A* regulates mitosis by inhibiting the APC-Cdc20 complex during prometaphase. Thus we analyzed the relation between *RASSF1A* methylation and proliferation of pituitary adenomas. Methylated tumors had a higher Ki-67 LI than unmethylated tumors. In addition, a higher Ki-67 LI was more frequently observed in methylated tumors than unmethylated tumors, although these differences did not reach statistical significance. It will be interesting to widely investigate this phenomenon in other tumors.

RASSF1C is another main isoform of *RASSF1*. In the present study, *RASSF1C* did not show hypermethylation and its mRNA was detected in similar amounts in all samples analyzed. Our results are same as those found in lung and breast tumors.^{12,24} However, a recent study demonstrated that *RASSF1C* also functions as a TSG, inducing growth inhibition in a prostate cell line LNCaP, a renal cell carcinoma line KRC/Y and in SCID mice; moreover, mutation or loss of expression of *RASSF1C* is associated with increased tumor growth *in vivo*. In addition, *RASSF1C* was shown to induce cell cycle arrest in KRC/Y cells.⁴⁰ These findings indicate that

RASSF1C may serve as a tumor suppressor gene and could have tissue-specific effects. Our data provide evidence that the methylation of the *RASSF1A* promoter is a specific and functionally important event in the expression of this TSG that is not globally related to other TSGs, including its closely situated homologue *RASSF1C*.

For altered expression of a gene, both alleles of a TSG need to be inactivated in the classical and revised two-hit hypothesis.^{2,41} *RASSF1A* inactivation accomplished via hypermethylation has been observed in many different kinds of cancers with frequent allelic imbalance at 3p21.3. In some of them, *RASSF1A* promoter methylation has been associated with LOH,^{36,42} but it has been found less frequently in other types of cancer.¹⁴ Most recently, methylation of *RASSF1A* has been identified in malignant cutaneous melanoma, medulloblastoma, and hepatocellular carcinoma that have rare LOH at 3p21.3.^{22,43,44} In addition, mutational inactivation of this gene is very rare.¹² These data suggested that promoter hypermethylation is the major mechanism for inactivation of *RASSF1A* in human tumors. Thus, it is not surprising that *RASSF1A* methylation has been identified in a considerable proportion of pituitary adenomas and correlated with loss or reduction of mRNA expression. In this study, MSP detected only unmethylated alleles in seven tumors and these tumors showed completely undetectable mRNA expression. Furthermore COBRA and bisulfite sequencing confirmed that there were only methylated alleles in these tumors. Owing to the rarity of LOH at 3p21.3 in pituitary adenomas,⁴⁵ *RASSF1A* inactivation caused by methylation of both alleles may be a critical event in pituitary tumorigenesis. In addition, 11 tumors had absent or significantly reduced *RASSF1A* expression despite the presence of both methylated and unmethylated alleles. A variety of possibilities may explain these data. First, it is possible that *RASSF1A* may belong to the group of haploinsufficient tumor suppressor genes that promote tumor formation through the inactivation of only one allele.⁴⁶ Alternatively, silencing could be due to other mechanisms, such as a separate genetic lesion to inactivate an unmethylated allele²⁰ or lack of transactivation factors. Another possibility is that nontumorous cells contaminating the frozen sample may contribute to the presence of unmethylated bands in MSP and detectable levels of mRNA. This possibility must be considered since MSP analysis is a very sensitive method that can detect a methylated gene at 0.1% dilution.²⁸ Therefore, unmethylated genes in nontumorous cells can be easily detected. Owing to our COBRA and bisulfite sequencing results, nontumorous cells contaminating the frozen sample may be the main reason for the unmethylated bands detected by MSP in these tumors. Also, bisulfite sequencing data showing complete or nearly complete methylation in all CpG islands, excluding the possibility that partial methylation could cause gene

expression in the tumor cells despite hypermethylation pattern in MSP.^{47,48}

The results presented here suggest that promoter hypermethylation of the *RASSF1A* gene has a critical role in pituitary tumor pathogenesis. The *RASSF1A* gene probably has a fundamental role as a tumor suppressor gene at an early stage of pituitary tumorigenesis and also may be involved in tumor proliferation and aggressiveness.

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