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Reduction of *GSTP1* expression by DNA methylation correlates with clinicopathological features in pituitary adenomas

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π-Class glutathione-*S*-transferase (*GSTP1*) located on chromosome 11q13 encodes a phase II metabolic enzyme that detoxifies reactive electrophilic intermediates. GSTP1 plays an important role in the protecting cells from cytotoxic and carcinogenic agents and is expressed in normal tissues at variable levels in different cell types. Altered GSTP1 activity and expression have been reported in many tumors and this is largely due to *GSTP1* DNA hypermethylation. The role of GSTP1 in pituitary tumorigenesis has not been investigated. In this study, we evaluated the GSTP1 expression level and *GSTP1* DNA methylation status in a series of pituitary adenomas. Using immunohistochemistry, we identified expression of GSTP1 in all of the various normal hormone-producing adenohypophysial cell types. In pituitary adenomas, loss or reduced expression of GSTP1 was detected in 27 of 53 tumors (50.9%). Expression of GSTP1 was significantly lower in invasive adenomas than in noninvasive adenomas ($P<0.05$). Using methylation-specific polymerase chain reaction (MS-PCR), *GSTP1* DNA promoter hypermethylation was detected in adenomas (38 of 53, 71.7%) but not in normal tissues. *GSTP1* methylation was more frequent in grade II, III, and IV tumors (66.7, 85, and 83%, respectively) than in grade I tumors (33%, $P<0.05$). In addition, the frequency of *GSTP1* methylation was higher in invasive tumors (85%) than in noninvasive tumors (59%; $P<0.05$). Methylation status correlated with significant downregulation of GSTP1 expression; the frequency of *GSTP1* methylation was higher in tumors with reduced-GSTP1 expression (85%) than in tumors with normal or high GSTP1 expression (54%; $P<0.05$).These data indicate that GSTP1 inactivation through CpG hypermethylation is common in pituitary adenomas and may contribute to aggressive pituitary tumor behavior.
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Pituitary adenomas are common intracranial neoplasms comprising 10–15% of diagnosed brain tumors. They may arise from any of the five differentiated cell types within the adenohypophysis, and reflecting their cellular origin, frequently synthesize and secrete their respective hormones. They can cause mood disorders, sexual dysfunction, infertility, obesity, visual disturbances, hypertension, diabetes mellitus, and accelerated heart disease.[1] However, the molecular pathway leading to pituitary tumorigenesis is one of the challenges of the endocrine oncology. While genetic events such as mutation, deletion, or rearrangement are relatively rare in pituitary adenomas, aberrant methylation-inducing tumor suppressor gene silencing is recognized as an important mechanism contributing to pituitary tumorigenesis.[1,2] Hypermethylation of gene promoter regions has been implicated in the inactivation of several genes, including *p16/CDKN2A*, *RB1*, *DAPK*, *GADD45γ*, *RASSF1A*, *E-cadherin*, *H-cadherin*, *Ikaros,* and *FGFR2*.[3–11]

GSTP1 encodes glutathione-*S*-transferase-π, a member of a family of enzymes, the glutathione-*S*-transferases (GSTs) that function as dimers

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composed of subunits from five main classes: α, μ, π, σ, and θ. GSTs are phase 2 enzymes that catalyze the conjugation of glutathione with electrophilic and hydrophobic compounds including carcinogens, natural toxins, and exogenous drugs.[12–14] GSTs are believed to play an important role in protecting cells from cytotoxic and carcinogenic agents. GSTP1 is expressed in normal tissues at various levels in different cell types.[15] *GSTP*-null mice show an increased risk of skin tumorigenesis induced by carcinogens,[16] suggesting that lack of GSTP1 expression increases susceptibility to cancer. Abnormal GSTP1 activity and expression and *GSTP1* DNA hypermethylation have been identified in prostate, breast, liver, renal, and endometrial carcinomas.[17–20] However, GSTP1 expression and *GSTP1* DNA methylation in pituitary adenomas has not been examined.

In this study, we evaluated the expression level and methylation status of GSTP1 in normal pituitary glands and pituitary adenomas. We performed immunohistochemical staining to determine GSTP1 protein expression and methylation-specific polymerase chain reaction (MS-PCR) to evaluate methylation of its promoter. Protein expression and epigenetic alterations were then correlated with clinicopathological parameters of the patients.

Materials and methods

Human Pituitary Tissues and Adenomas

Three normal human adenohypophyses were obtained at autopsy from patients with no evidence of endocrine abnormality; they were examined histologically to exclude the possibility of incidental pathology. A total of 53 pituitary adenomas 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 clinical, radiological, histological, and immunohistochemical features (Table 1). There were 33 clinically functioning tumors (17 somatotroph adenomas, 1 mammosomatotroph adenoma, 10 lactotroph adenomas, 4 corticotroph adenomas associated with Cushing's disease, and 1 thyrotroph adenoma), and 20 clinically nonfunctioning adenomas (4 silent corticotroph adenomas, 12 gonadotroph adenomas, 3 silent subtype 3 adenomas, and 1 null cell adenoma). Tumor size and invasiveness were defined on the basis of preoperative radiological investigations and operative findings and with a modified Hardy's classification. 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 were 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, the 53 tumors included six tumors of grade I, 21 tumors of grade II, 20 tumors of grade III, and six tumors of grade IV (27 noninvasive and 26 invasive adenomas; Table 1, 5).

Table 1 Clinical and pathologic characteristics in 53 pituitary adenomas

	No. of cases	*Tumor size*		*Invasiveness*	
		Macroadenoma	*Microadenoma*	*Invasive*	*Noninvasive*
Patients (*n*; %)	53	47 (88.7)	6 (11.3)	26 (49.0)	27 (50.9)
Gender (n; %)					
Male	25	25 (100.0)	0 (0.0)	11 (44.0)	14 (56.0)
Female	28	22 (78.6)	6 (21.4)	15 (53.6)	13 (46.4)
Functional					
GH	17	15 (88.2)	2 (11.8)	8 (47.0)	9 (52.9)
GH/PRL	1	1 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)
PRL	10	7 (70.0)	3 (30.0)	5 (50.0)	5 (50.0)
ACTH	4	3 (75.0)	1 (25.0)	1 (25.0)	3 (75.0)
TSH	1	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)
Total	33	27 (81.8)	6 (18.2)	15 (45.4)	18 (54.5)
Nonfunctional					
ACTHs	4	4 (100.0)	0 (0.0)	4 (100.0)	0 (0.0)
FSH/LH	12	12 (100.0)	0 (0.0)	3 (25.0)	9 (75.0)
Null cell	1	1 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)
Type 3	3	3 (100.0)	0 (0.0)	3 (100.0)	0 (0.0)
Total	20	20 (100.0)	0 (0.0)	11 (55.0)	9 (45.0)

ACTH, corticotroph adenoma; ACTHs, silent corticotroph adenoma; FSH/LH, gonadotroph adenoma; GH, somatotroph adenoma; GH/PRL, mammosomatotroph adenoma; null cell, null-cell adenoma; PRL, lactotroph adenoma; subtype 3, silent subtype 3 adenoma; TSH, thyrotroph adenoma.

Immunohistochemical Analysis of GSTP1 Expression in Normal Pituitary Tissues

A double-staining technique was performed to examine the expression of GSTP1 protein in three normal pituitary tissues following a modification of the method of Hsu and Soban.[21] Briefly, the GSTP1 protein was stained using the labeled streptavidin biotin (LSAB) method. After deparaffinization and antigen retrieval using an autoclave oven technique, sections were incubated at 4°C overnight with antibody that recognizes GSPT1 (DakoCytomation, Carpinteria, 1:100 dilution). The biotinylated link antibody and peroxidase-labeled streptavidin were applied for 1 h each at room temperature. Sections were washed thoroughly in phosphate buffered saline (PBS) between each of the immunostaining procedures. Antigen–antibody complexes were detected using the cobalt-3,3′-diaminobenzidine (Co-DAB) yielding a blue–black color. After washing in PBS, the sections were then incubated in 0.005% biotin solution. Subsequently, the localization of the pituitary hormones in the same sections was detected by LSAB method with antihormone antibodies (Table 2), which yielded a brown color using DAB. Sections incubated in PBS without primary antibody served as negative controls.

Immunohistochemical Analysis of GSTP1 Expression in Pituitary Adenomas

GSTP1 and Ki-67 antigen immunolocalization based on the LSAB method were performed on sections from representative blocks of paraffin-embedded tissues used for pathology diagnosis. After deparaffinization and antigen retrieval using an autoclave oven technique, sections were incubated at 4°C overnight with Ki-67 antigen mouse monoclonal antibody (Table 2) or with an antibody that recognizes GSTP1 (Table 2). Antigen–antibody complexes were detected using the DAB reaction. The slides were counterstained lightly with hematoxylin or 1% methyl green and mounted for microscopic examination. Sections incubated in PBS without the primary antibody served as negative controls.

Quantification of GSTP1 and Ki-67 Antigen Immunoreactivity

Each slide was examined by an observer blinded to the diagnosis and clinicopathologic data; and reviewed and confirmed by a second blinded observer. Any intensity of cytoplasmic staining was considered to present a positive stain for GSTP1. A total of 500–1000 cells were counted and the percentage of GSTP1-stained tumor cells was scored on a scale of 0–4 (0: no expression; 1+: 1–5%; 2+: 5–20%; 3+: 20–50%; 4+: >50%). The Ki-67 antigen labeling index (LI) was determined by counting the number of positive cells in a total of 500–1000 tumor cells observed in several representative high-power fields ($\times 400$).

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.

Methylation-specific PCR

The promoter methylation status of *GSPT1* was investigated by methylation-specific PCR (MSP) assay as described previously.[18] Two sets of *GSPT1* promoter-specific primers described by Esteller *et al*[18] 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. 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 direct sequencing using the Nucleo Spin® Extract Kit (Macherey-nagel, Düren, Germany). Cycle sequencing was performed using the BigDye Terminator V1.1 Cycle Sequencing Kit

Table 2 List of primary antibodies used

Anti-	*Source*	*Clone*	*Dilution*	*Pretreatment*
GSTP1	DakoCytomatin, Capinteria, CA, USA	Polyclonal	1:100	None
GH	DakoCytomatin, Capinteria, CA, USA	Polyclonal	1:400	None
ACTH	DakoCytomatin, Capinteria, CA, USA	Polyclonal	1:250	None
PRL	NeoMarkers Inc., CA, USA	SPM108	1:200	None
FSH	Novocastra Lab., Ltd, UK	INN-hFSH-60	1:100	Protease K
TSH	DakoCytomatin, Capinteria, CA, USA	0042	1:100	None
LH	NeoMarkers Inc., CA, USA	LH01	1:500	None
Ki-67	DakoCytomatin, Glostrup, Denmark	MIB-1	1:100	None

(Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

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

Expression of GSTP1 in Normal Pituitary Tissues and Pituitary Adenomas

In three normal adenohypophyseal samples, GSTP1 exhibited cytoplasmic reactivity with moderate density (10–20%; 2+); no membrane or nuclear localization was identified (Figure 1a). Using double staining, GSTP1 expression colocalized with each of the adenohypophysial hormones including GH, PRL, ACTH, FSH, TSH, and LH (Figure 1b–g). These results imply that GSTP1 may protect all of the various hormone-producing cell types in normal pituitary gland against carcinogens.

In pituitary adenomas, GSTP1 expression was lost in 38% (20 of 53 tumors), was significantly reduced in 13% (7 of 53 tumors), and was normal or high in 49% (26 of 53 tumors) where staining was 2+ in 17, 3+ in 8 and 4+ in 1 (Figure 2A, Table 3).

Loss or reduced expression of GSTP1 was observed in GH cell adenomas (seven cases, 41.2%), in PRL cell adenomas (nine cases, 90%), ACTH cell adenomas with Cushing's syndrome (one case, 25%) and silent ACTH cell adenomas (four cases, 100%), but was found rarely in FSH/LH cell adenomas (two cases, 16.7%) (Table 3). The expression of GSTP1 was significantly lower in invasive adenomas than in noninvasive adenomas (Figure 3a) and the frequency of adenomas with GSTP1 expression was higher in invasive adenomas than in noninvasive adenomas (Table 3). The reduced expression of GSPT1 was not related to patient age, gender, or tumor size (data not shown).

Methylation Status of *GSTP1* in Normal Pituitary Tissues and Pituitary Adenomas

We used MSP to investigate the promoter methylation of *GSTP1* in three normal pituitary tissues and 53 pituitary adenomas. The methylation region included 19 CpG sites that were previously found by MSP to be associated with reduced-GSTP1 expression (Figure 2B-a). Hypermethylation of the promoter region of *GSTP1* was detected in 38 of 53 (71.7%) pituitary adenomas, but not in three normal pituitary tissues (Figure 2B-b, Table 4) suggesting that *GSTP1* promoter hypermethylation is tumor-specific in the pituitary. Methylated patterns of *GSTP1* were found in all major types of pituitary adenomas. Unmethylated bands in MSP were detected in all four normal tissues and in all adenoma samples (Table 4).

There was no significant correlation between *GSTP1* methylation and patient age. Interestingly, *GSTP1* methylation was detected more frequently in grade II, III, and IV tumors (66.7, 85, and 83%, respectively) than in grade I tumors (33%, $P<0.05$). In addition, the frequency of *GSTP1* methylation was higher in invasive tumors (84.6%) than in noninvasive tumors (63%; $P<0.05$, Table 5). The difference in frequency between tumor type and patient gender was not statistically significant. The Ki-67 LI was also not significantly related to *GSTP1* methylation in pituitary adenomas (data not shown).

The specificity of MSP was confirmed by direct sequencing. Six unmethylated MSP products from two normal tissues and four tumors, and six methylated MSP products from six tumors were directly sequenced. In unmethylated MSP products, all cytosine nucleotides including those in the CpG islands were changed to thymine as a result of bisulfite modification. However, in methylated MSP products, cytosine nucleotides in the CpG islands remained cytosine (Figure 2B-c).

Correlation between *GSTP1* Promoter Hypermethylation and Loss or Significant Reduction of GSTP1 Expression

We analyzed the relationship between GSTP1 protein expressions and *GSTP1* methylation statues in three normal pituitary tissues and 49 pituitary adenomas. Three normal pituitary tissues with unmethylated alleles showed moderate (2+) GSTP1 protein expression. Methylation was detected in 23 of 27 tumors (87%) with reduced expression of GSTP1 (Table 5, $P<0.05$). Furthermore, GSTP1 expression levels were significantly lower in pituitary tumors with *GSTP1* methylation than in pituitary adenomas without *GSTP1* methylation (Figure 3b, $P<0.05$). Overall, there was a significant correlation between *GSTP1* promoter hypermethylation and reduced-GSTP1 expression.

Discussion

Previous studies provided a line of evidences for the presence of GSTP1 protein in various normal human tissues using biochemical methods and immunohistochemistry.[22,23] GSTP1 is strongly expressed in the epithelial cells of the urinary, digestive, and respiratory tracts, these major systems being involved in elimination of toxic substances from the human body. In endocrine organs, GSTP1 is expressed in follicular cells of the thyroid and both cortex and medulla of adrenal glands. In pancreas,

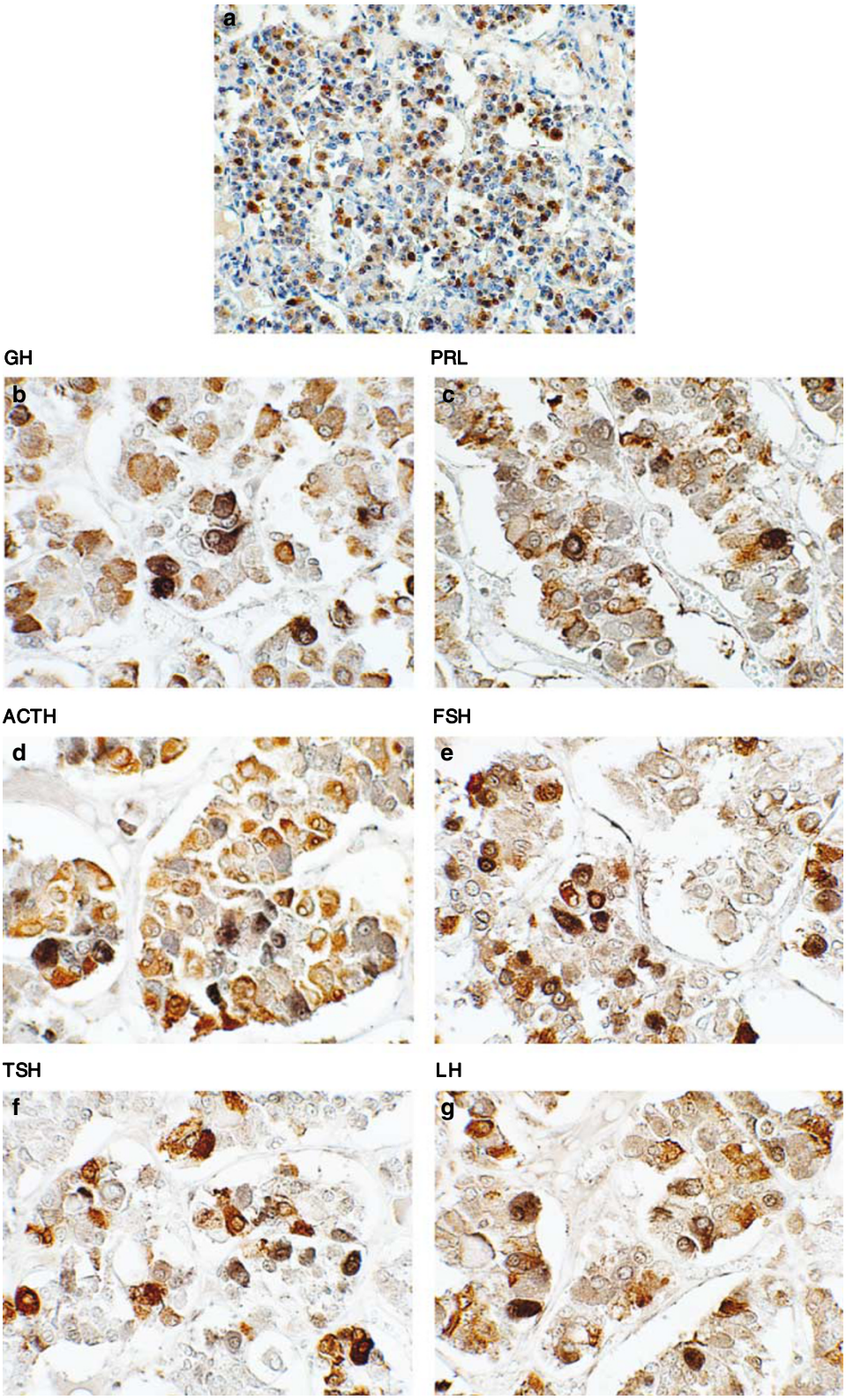

Figure 1 (**a**) GSTP1 immunostaining in normal pituitary cells showed cytoplasmic immunoreaction. (**b–g**) Double staining for GSTP1 and hormones. GSTP1 is localized by a blue–back color and hormones are identified by a brown chromogen. GSTP1 shows immunoreaction in each type of hormone-secreting adenohypophysial cell.

GSTP1 is expressed in ducts but not in acini or islets.[23] GSTP1 also is expressed in other endocrine-related organs such as the breast, prostate, uterus, and placenta.[23] The expression of GSTP1 in pituitary gland has not been reported. Here, we performed a detailed study using double immuno-

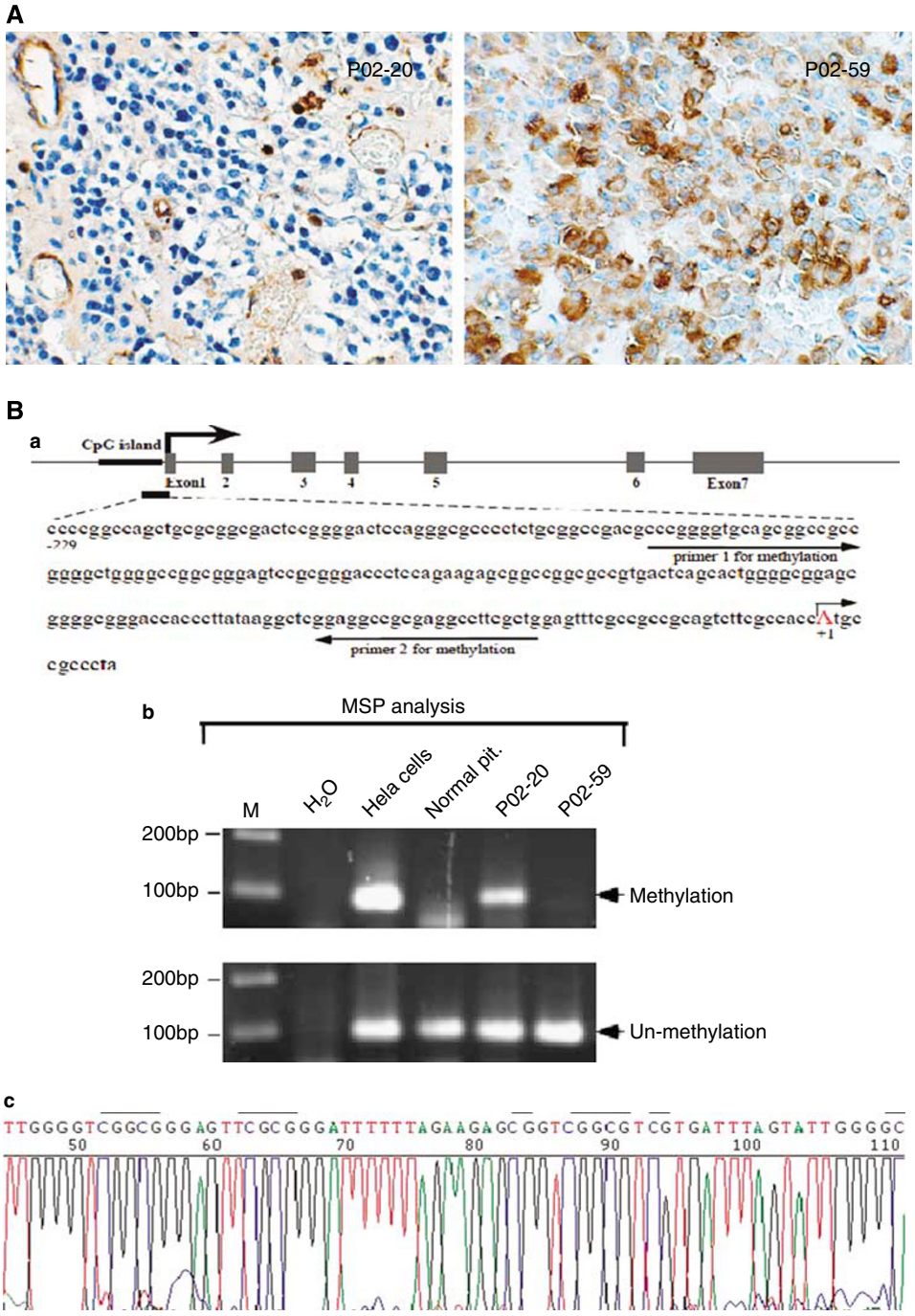

Figure 2 (**A**) GSTP1 immunostaining in pituitary tumors: P02-20 showed negative immunoreaction; P02-59 showed positively cytoplasmic immunoreaction. (**B**) Analysis of *GSTP1* DNA methylation in normal pituitary and pituitary tumors. (**a**) Nucleotide sequence of the CpG island region in the *GSTP1* promoter (before sodium bisulfite conversion). Total 19 CpG sites were analyzed by MSP. (**b**) The methylation status of the *GSTP1* promoter was analyzed by MSP in normal pituitary tissues and pituitary adenomas (P02-20, P02-59). In a *GSTP1*-negative sample (P02-20), *GSTP1* DNA methylation was detected; in a *GSTP1*-positive sample (P02-59), there are unmethylated bands. (**c**) Direct sequence of MSP methylation-specific products of *GSTP1* from pituitary adenomas. None of the CpG dinucleotides were converted to TpG dinucleotides by bisulfite treatment. The only remaining cytosines in the MSP-amplified DNA segment showing methylated cytosines that are 5′ to guanosines are indicated (underlined).

histochemical labeling to examine the distribution of GSTP1 in the normal human pituitary gland. GSTP1 is strongly expressed in about 10–20% of endocrine cells in the adenohypophysis; GSTP1 protein colocalizes with all hormones including GH, PRL, ACTH, TSH, FSH, and LH. Thus, GSTP1 may play an important role in pituitary cells irrespective of type of hormone produced. As GSTs may be engaged in the intracellular transport and metabolism of steroid hormones,[24] it is suggested that

Table 3 GSTP1 expression in pituitary adenomas and normal pituitary tissues

	No. of cases	*Expression intensity of GSTP1*					*Negative/low expression (≦1+)*	*Normal/high expression (≧2+)*
		−	*1+*	*2+*	*3+*	*4+*		
Patients (*n*; %)	53	20	7	17	8	1	27 (50.9)	26 (49.1)
Functional								
GH	17	6	1	9	1	0	7 (41.2)	10 (58.8)
GH/PRL	1	0	1	0	0	0	1 (100.0)	0 (0)
PRL	10	8	1	0	1	0	9 (90.0)	1 (10.0)
ACTH	4	0	1	1	2	0	1 (25.0)	3 (75.0)
TSH	1	0	1	0	0	0	1 (100.0)	0 (0)
Total	33	14	5	10	4	0	19 (57.6)	14 (42.4)
Nonfunctional								
ACTHs	4	4	0	0	0	0	4 (100.0)	0 (0)
FSH/LH	12	1	1	6	3	1	2 (16.7)	10 (83.3)
Null cell	1	0	0	0	1	0	0 (0)	1 (100.0)
Type 3	3	1	1	1	0	0	2 (66.7)	1 (33.3)
Total	20	6	2	7	4	1	8 (40.0)	12 (60.0)
Invasiveness								
Invasive	26	13	4	7	2	0	17 (65.4)	9 (34.6)[a]
Noninvasive	27	7	3	10	6	1	10 (37)	17 (63)[a]
Normal pituitary	3	0	0	3	0	0	0 (0)	3 (100.0)

[a]$P<0.05$.

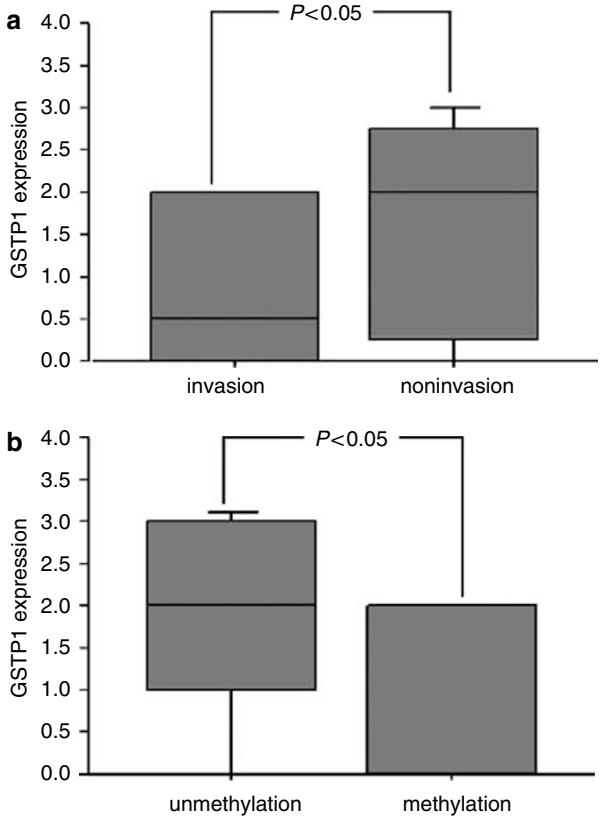

Figure 3 (**a**) GSTP1 expression levels were significantly lower in invasive pituitary adenomas than in noninvasive pituitary adenomas ($P<0.05$). (**b**) GSTP1 expression levels were significantly lower in pituitary tumors with methylation pattern than in pituitary adenomas without methylation ($P<0.05$).

Table 4 *GSTP1* methylation in pituitary adenomas and normal pituitary tissues

	No. of cases	*GSTP1 methylation*	
		Methylated	*Unmethylated*
Patients (*n*; %)	53	38 (71.7)	15 (28.3)
Gender n; %)			
Male	25	19 (76.0)	6 (24.0)
Female	28	19 (67.9)	9 (32.1)
Functional			
GH	17	13 (76.5)	4 (23.5)
GH/PRL	1	1 (100.0)	0 (0.0)
PRL	10	8 (80.0)	2 (20.0)
ACTH	4	3 (75.0)	1 (25.0)
TSH	2	1 (50.0)	1 (50.0)
Total	34	26 (76.5)	8 (23.5)
Nonfunctional			
ACTHs	4	4 (100.0)	0 (0.0)
FSH/LH	11	6 (54.5)	5 (45.4)
Null cell	1	0 (0.0)	1 (100.0)
Type 3	3	2 (66.7)	1 (33.3)
Total	19	12 (63.2)	7 (36.8)
Normal pituitary	3	0 (0.0)	3 (100.0)

GSTP1 may have a possible role in hormone transport or metabolism.

Approximately 51% pituitary adenomas showed negative or reduced expression of GSTP1. The downregulated expression of GSTP1 was significant related to tumor invasion. However, it should be

Table 5 Correlation of GSTP1 methylation and tumor aggressiveness and GSTP1 expression

	No. of cases	*GSTP1 methylation*		P
		Methylated	*Unmethylated*	
Tumor grade	53			<0.05
I	6	2 (33.3)	4 (66.7)	
II	21	14 (66.7)	7 (33.3)	
III	20	17 (85.0)	3 (15.0)	
IV	6	5 (83.3)	1 (16.7)	
Invasiveness				<0.05
Invasive	26	22 (84.6)	4 (15.4)	
Noninvasive	27	16 (59.2)	11 (40.7)	
GSTP1 expression	49			<0.05
≦1+	27	23 (85.2)	4 (14.8)	
≧2+	22	12 (54.5)	10 (45.5)	

noted that reduced expression of GSTP1 was detected in a high proportion of silent ACTH and PRL adenomas (100, 90%, respectively) but only in 16.7% of gonadotroph adenomas. This interesting phenomenon implies that GSTP1 may be involved in pituitary tumorigenesis through multimechanism. GSTP1 is known to bind steroid hormones noncovalently. It allows GSTP1 protein to minimize the effects of short-term fluctuations in extracellular hormone levels.[12] A role of estrogens in the development of PRL adenomas has been detected by several investigations. *In vitro* estrogen stimulates PRL secretion and *in vivo* estrogen induces PRL adenomas in human and in animal models.[25] Thus loss or reduction of GSTP1 may enhance the effect of estrogens in progression of PRL adenomas. It is very meaningful and consequent that reduced expression of GSTP1 was frequently found in PRL adenomas. By the same ability, GSTP1 may impair the estrogen-induced negative feedback of gonadotropin secretion. Interestingly, one study demonstrated that estrogen-induced negative feedback of gonadotropin secretion is disrupted in patients with gonadotroph adenomas.[26] Thus, we hypothesize that normal or high expression of GSTP1 may contribute to gonadotroph adenoma development through regulation of estrogen-induced negative feedback of gonadotropin secretion. Anyway, further investigation is necessary to profile the exact role of GSTP1 in each type of pituitary adenoma.

Hypermethylation of CpG islands in the *GSTP1* promoter and its associated gene silencing have been reported in a variety of tumors.[18] In the current study, we demonstrate *GSTP1* promoter hypermethylation in 72% of pituitary adenomas including all the major clinically functioning and hormonally inactive types. There was no significant correlation between *GSTP1* methylation and patient age, thus methylation of *GSTP1* in pituitary adenomas is not attributable to the aging process, as has been described for other genes.[27] *GSTP1* methylation was significantly associated with loss or reduction of protein expression in these pituitary adenomas. Noticeably, *GSTP1* methylation was frequently detected in pituitary tumors that exhibited no GSTP1 expression. In contrast, the *GSTP1* promoter was unmethylated and GSTP1 expression level was high in normal pituitary tissues. As *GSTP1* methylation is tumor specific and has high frequency, it may be used as a biomarker in diagnosis of pituitary adenomas. The possibility of *GSTP1* methylation as molecular diagnostic tool in prostate cancer has been wildly discussed. In prostate cancer patients, *GSTP1* methylation was successfully detected in body fluids such as serum, urine, and ejaculates.[28] Thus, to detect *GSTP1* methylation in serum from pituitary adenoma patients is very necessary for determining its true value in the diagnostic clinical setting.

Previous studies have shown an association of *GSTP1* methylation with tumor invasion, tumor size, sentinel lymph node metastasis, and progression in several tumor types including endometrial carcinoma, breast cancer, and prostate cancer.[19,29,30] In this study, we demonstrate that hypermethylation of *GSTP1* occurs more frequently in grade II, III, and IV adenomas (macroadenomas) and invasive adenomas than in grade I adenomas (microadenomas) and noninvasive adenomas. These findings suggest that in addition to a role in tumorigenesis, *GSTP1* methylation may also relate to the progression of pituitary adenoma. However, the number of grade I and IV tumors in this series is small and mechanisms related to this risk are still unknown.

Generally, *GSTP1* has several roles in tumorigenesis. First, *GSTP1* might act like a tumor suppressor gene, which leads to tumor growth, when inactivated. Adler *et al*[31] have reported that π-class GSTs can interfere with *N*-terminal c-Jun kinase signaling. Second, *GSTP1* might act like a caretaker gene, which leads to additionally somatic genome alterations that promote tumor growth, when inactivated.[32] GSTP1, like other GSTs, can catalyze the detoxification of oxidants and electrophiles that threaten genome damage.[12] For example, mice carrying disrupted *GSTP* alleles display enhanced skin tumorigenesis on exposure to 7,12-dimethylbenzanthracene.[33] Interestingly, GSTP1 is able to bind steroid hormones noncovalently, allowing the protein to act as an intracellular buffer, minimizing the effects of short-term fluctuations in extracellular hormone levels.[34] It is noteworthy that silencing of the *GSTP1* gene appears to be restricted to prostate, breast, kidney, and endometrial carcinomas, which are related to steroid hormone exposure. Thus, epigenetic silencing of *GSTP1* by promoter methylation might similarly facilitate the carcinogenic action of estrogens as endogenous tumor initiators.

How can loss of GSTP1 expression by promoter hypermethylation be involved in the development of pituitary adenomas? On the basis of our study,

GSTP1 may act as a tumor suppressor gene and/or caretaker gene to contribute to pituitary tumorigenesis. Although loss of GSTP1 did not relate to high expression of the proliferation marker Ki-67, methylation of *GSTP1* induced GSTP1 downregulation that correlates with pituitary adenoma aggressiveness. These data imply that *GSTP1* may work as a tumor suppressor gene in pituitary tumor development. On the other hand, the majority of pituitary adenomas develop from transformed cells dependent on hormonal stimulation for tumor promotion.[35] GHRH upregulation might promote cell proliferation in somatotrophs that are already transformed, or GHRH-induced hyperplasia might provide an environment for cell transformation. Untreated hypothyroidism is associated with a spectrum of hyperplasia-to-neoplasia transition—consistent with the idea that TRH stimulation can lead to thyrotroph adenoma formation. In rat models, estrogen treatment is associated with increased expression of vascular endothelial growth factor (VEGF), pituitary tumor transforming gene (*PTTG*), and galanin.[1] On the basis of our result that GSTP1 is frequently silenced in pituitary adenomas, and especially in prolactinomas, we hypothesize that loss of *GSTP1* could be one basis of hormone-induced pituitary tumorigenesis. However, additional confirmation of such a hypothesis is necessary.

To conclude, we identified GSTP1 protein expression in all hormone-producing adenohypophysial cells. Furthermore, we demonstrated reduced-GSTP1 expression and hypermethylation of the *GSTP1* gene in a significant proportion of pituitary adenomas and we found that reduced expression and gene methylation were related to pituitary tumor invasiveness and tumor grade. Hypermethylation of the *GSTP1* gene may be a flexible and dynamic mechanism in the control of gene expression that plays a role in pituitary tumorigenesis and tumor progression.

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