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Tumor-specific downregulation and methylation of the *CDH13* (H-cadherin) and *CDH1* (E-cadherin) genes correlate with aggressiveness of human pituitary adenomas

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The gene products of *CDH13* and *CDH1*, H-cadherin and E-cadherin, respectively, play a key role in cell–cell adhesion. Inactivation of the cadherin-mediated cell adhesion system caused by aberrant methylation is a common finding in human cancers, indicating that the *CDH13* and *CDH1* function as tumor suppressor and invasion suppressor genes. In this study, we analyzed the expression of H-cadherin mRNA and E-cadherin protein in 5 normal pituitary tissues and 69 primary pituitary adenomas including all major types by quantitative real-time RT-PCR (qRT-PCR) and immunohistochemistry, respectively. Reduced expression of H-cadherin was detected in 54% (28/52) of pituitary tumors and was significantly associated with tumor aggressiveness ($P < 0.05$). E-cadherin expression was lost in 30% (21 of 69) and significantly reduced in 32% (22 of 69) of tumors. E-cadherin expression was significantly lower in grade II, III, and IV than in grade I adenomas ($P = 0.015$, $P = 0.029$, and $P = 0.01$, respectively). Using methylation-specific PCR (MSP), promoter hypermethylation of *CDH13* and *CDH1* was detected in 30 and 36% of 69 adenomas, respectively, but not in 5 normal pituitary tissues. Methylation of *CDH13* was observed more frequently in invasive adenomas (42%) than in non-invasive adenomas (19%) ($P < 0.05$) and methylation of *CDH1* was more frequent in grade IV adenomas compared with grade I adenomas ($P < 0.05$). Methylation of either *CDH13* or *CDH1* was identified in 35 cases (51%) and was more frequent in grade IV invasive adenomas than in grade I non-invasive adenomas ($P < 0.05$ and $P < 0.05$, respectively). Downregulation of expression was correlated with promoter hypermethylation in *CDH13* and *CDH1*. In conclusion, the tumor-specific downregulation of expression and methylation of *CDH13* and *CDH1*, alone or in combination, may be involved in the development and invasive growth of pituitary adenomas.
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Pituitary adenomas constitute about 10–15% of intracranial neoplasms. Despite the classification of these tumors as benign, a proportion of them invade surrounding structures including the sphe-noid sinus, the cavernous sinus, and even the brain. The pathogenetic mechanisms underlying pituitary adenoma formation and progression remain unclear. Mutations in classic oncogenes and tumor suppressor genes (TSGs) are only rarely found in these tumors.[1] Furthermore, the mechanisms responsible for aggressive behavior are also poorly understood.

The gene product of *CDH13*, namely H-cadherin, is a new unique member of the cadherin super-family. H-cadherin is anchored to the cell-surface membrane through a glycosyl phosphatidylinositol moiety and lacks the cytoplasmic domain unlike

other cadherins such as E-cadherin, N-cadherin, and P-cadherin.[2] Recent studies have highlighted the role of *CDH13* as a TSG in lung, breast, ovarian, bladder, colorectal, esophageal, gastric, cutaneous, and pancreatic cancers.[3–10] Furthermore, downregulation of H-cadherin due to hypermethylation in the promoter region of *CDH13* gene appears to be related to the tumorigenesis and invasiveness of these cancers. However, expression of H-cadherin and the methylation status of the *CDH13* in pituitary tissue and pituitary adenomas have not been evaluated to date.

E-cadherin, the gene product of *CDH1*, plays a key role in cell–cell adhesion. Decrease or loss of E-cadherin expression accompanied by *CDH1* promoter methylation has been reported in many human cancers.[11] In pituitary adenomas, E-cadherin expression might play a variety of roles in different tumor subtypes. Our previous studies showed that the decreased expression of E-cadherin was related to aggressive behavior of prolactinomas and was associated with the formation of fibrous bodies in GH cell adenomas.[12,13] However, methylation status of the *CDH1* promoter has not been examined in pituitary adenomas and the relation between E-cadherin expression and invasion by pituitary adenomas is unclear.

Methylation of CpG islands in gene promoter regions is associated with aberrant silencing of transcription as an alternative mechanism for TSG inactivation to gene deletion and mutations. Thus, it is a frequently acquired epigenetic event in the pathogenesis of many human malignancies.[14] There is increasing evidence of aberrant promoter methylation of TSGs in the pathogenesis of pituitary adenomas, although some of them are tumor subtype specific.[15–20] The *p16/CDKN2A* and *RB1* gene methylation with tumor subtype specificity were described in pituitary tumors.[15–17] Preferential loss of death-associated protein kinase (DAPK) expression in invasive pituitary tumors has been reported to be associated with CpG island methylation.[18] Methylation-associated gene silencing of the *GADD45γ* gene, a negative regulator of cell growth, also has been found in human pituitary tumors and a mouse pituitary tumor cell line AtT20.[19] Most recently, hypermethylation of *RASSF1A* and resultant alteration of *RASSF1A* expression have been detected in pituitary adenomas of all types.[20]

These results promoted us to investigate the roles of *CDH13* and *CDH1* in human pituitary tumorigenesis and tumor behavior. In this study, the expression of H-cadherin and E-cadherin was examined by quantitative real-time RT-PCR (qRT-PCR) and immunohistochemistry, respectively. We also examined the frequency of methylation of the *CDH13* and *CDH1* gene promoters in various types of pituitary adenomas using methylation-specific PCR (MSP). The results were compared to the clinicopathologic parameters of these pituitary adenomas.

Materials and methods

Human Pituitary Tissues and Adenomas

Five normal human adenohypophyses were obtained at autopsy from patients with no evidence of endocrine abnormality at Tokushima University Hospital (Tokushima, Japan); they were examined by hematoxylin–eosin stain and immunocytochemistry to exclude the possibility of incidental tumors. Sixty-nine pituitary adenoma specimens were obtained at the time of surgery at Tokushima University Hospital and Toranomon Hospital (Tokyo, Japan). All samples were frozen and stored at $-80°C$. Tumors were characterized based on the clinical, radiological, histological, and immunohistochemical features (Table 1).[21] There were 45 clinically functional tumors (24 somatotroph adenomas, 2 mammosomatotroph adenomas, 12 lactotroph adenomas, 4 corticotroph adenomas associated with Cushing's disease, and 3 thyrotroph adenomas) and 24 clinically non-functioning adenomas (6 silent corticotroph adenomas, 14 gonadotroph adenomas, 3 silent subtype 3 adenomas and 1 null cell adenoma characterized by 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.[22] Grade I (microadenomas, <1 cm in diameter) and grade II (enclosed macroadenomas with or without suprasellar extension, ≥1 cm in diameter) tumors are defined as non-invasive. Grade III (local invasion of sphenoid and/or cavernous sinus) and grade IV (central nervous system/extracranial spread with or without metastasis) tumors were considered to be invasive. Thus, 69 tumors included 9 tumors of grade I, 27 tumors of grade II, 25 tumors of grade III, and 8 tumors of grade IV (36 non-invasive and 33 invasive adenomas; Table 1). None of the tumors examined in this study had evidence of postoperative recurrence

Total RNA and DNA Extraction

Total RNA and DNA were extracted from fresh frozen tissue samples using the Isogen (Nippon Gene, Toyama, Japan) and Qiagen DNeasy Tissue Kit (Qiagen, Stanford, CA, USA), respectively, following the manufacturer's protocol.

Quantitative Real-Time RT-PCR Analysis of *CDH13* Expression

Since no antibody is available to detect H-cadherin protein expression, we evaluated mRNA expression of *CDH13* in 52 pituitary adenomas for which mRNA was available. First-strand cDNA was synthesized from 1 μg of each purified RNA sample using

Table 1 Summary of *CDH13* and *CDH1* methylation in pituitary adenomas and normal pituitary glands

Variable	No.	CDH13 methylation		P	CDH 1 methylation		P
		+	−		+	−	
Patients	69	21 (30%)	48 (70%)		25 (36%)	44 (64%)	
Age (years)		43.6 ± 3.2	48.4 ± 2.3	0.25	47.7 ± 3.6	46.5 ± 2.2	0.75
Gender				0.28			0.60
Male	33	8 (24%)	25 (76%)		13 (40%)	20 (60%)	
Female	36	13 (36%)	23 (64%)		12 (33%)	24 (67%)	
Functional				0.45[a]			0.19[a]
GH	24	6 (25%)	18 (75%)		5 (21%)	19 (79%)	
GH/PRL	2	1 (50%)	1 (50%)		0	2 (100%)	
PRL	12	4 (33%)	8 (67%)		6 (50%)	6 (50%)	
ACTH	4	1 (25%)	3 (75%)		1 (25%)	3 (75%)	
TSH	3	2 (67%)	1 (33%)		2 (67%)	1 (33%)	
Total	45	14 (31%)	31 (69%)		14 (31%)	31 (69%)	
Non-functional							
ACTHs	6	3 (50%)	3 (50%)		1 (17%)	5 (83%)	
FSH/LH	14	2 (14%)	12 (86%)		7 (50%)	7 (50%)	
Subtype 3	3	1 (33%)	2 (67%)		2 (67%)	1 (33%)	
Null cell	1	1 (100%)	0		1 (100%)	0	
Total	24	7 (29%)	17 (71%)		11 (46%)	13 (54%)	
Normal	5	0	5 (100%)		0	5 (100%)	

ACTH, corticotroph adenoma; ACTHs, Silent corticotroph adenoma; FSH/LH, gonadotroph adenoma; GH, somatotroph adenoma; GH/PRL, mammosomatotroph adenoma; PRL, lactotroph adenoma; Subtype 3, silent subtype 3 adenoma; Null cell, null cell adenoma.
Normal: normal pituitary tissues.
[a]Functional *vs* non-functional.

ExScript™ RTase primed with random hexamers (*ExScript*™ RT-PCR kit; TaKaRa, Kyoto, Japan) according to the protocol recommended by the manufacturer. The gene expression levels of *CDH13* were then quantified using TaqMan technology on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Forster City, CA, USA). Gene-specific primers and probe of *CDH13* (assay ID Hs00169908_m1) were available as TaqMan Gene Expression Assays (Applied Biosystems). The 18S ribosomal RNA (18S rRNA) was amplified and was used as an endogenous control in the quantifications. The real-time PCR was performed in 20 μl reaction containing 1× premix Ex Taq, 1 × Target Assay Mix, ROX reference dye (TaKaRa), and 1 μl of first-strand cDNA from each sample as a template, using MicroAmp optical 96-well plates covered with MicroAmp optical caps (Applied Biosystems). The thermocycling conditions were 10 s at 95°C, and 40 cycles of 5 s at 95°C and 31 s at 60°C. All qRT-PCR experiments included a no template control and were performed in duplicate. Serial dilutions of cDNA from normal pituitary tissues were amplified in parallel as a control of amplification efficiency within each experiment and for the establishment of a standard curve for relative quantification. Expression of *CDH13* mRNA was normalized for *18S* rRNA as an internal reference. Relative expression levels were calculated as *CDH13/18S* rRNA in tumor and normal tissues, respectively.

Immunohistochemical Analysis of E-Cadherin Expression

E-cadherin immunolocalization using the labeled streptavidin–biotin method was performed on sections from representative blocks of paraffin-embedded tissues used for pathology diagnosis. Anti-E-cadherin mouse monoclonal antibody (1:500 dilution; Transduction Laboratories, Lexington, KY, USA) was used as in our previous study.[13] For positive controls, normal epidermis known to be positive for E-cadherin was used. Positive expression of E-cadherin was defined as exclusively membranous staining, as seen in normal epithelial cell of the epidermis. Both the intensity of staining and the percentage of positive tumor cells of E-cadherin in each specimen were considered in semi-quantitative assessment. The distribution of positive staining in the tumor was graded into a five-tier scoring system (0, no staining; 1+, 1–20%; 2+, 20–50%; 3+, 50–80%; and 4+, >80%). The intensity was assigned as weak (0), moderate (1+), or intense (2+). The scores were added together to obtain a total score that can range from 0 to 6. Tumors scoring 1, 2, and 3 were classified as the significantly reduced expression of E-cadherin.

Bisulfite Modification and Methylation-Specific PCR

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. Subsequently, the DNA promoter methylation status of the *CDH13* and *CDH1* gene was investigated by MSP assay as described previously.[23] Both specific primers for methylated and unmethylated promoters and annealing temperature applied were described in previous reports.[3,4,23] The primers for the methylated *CDH13* were (sense) 5'-TCGCGGGGTTCGTT TTTCGC-3' and (antisense) 5'-GACGTTTTCATTCA TACACGCG-3' (annealing temperature: 57°C, 243 bp product) and for the unmethylated form were (sense) 5'-TTGTGGGGTTTGTTTTTTGT-3' and (antisense) 5'-AACTTTTCATTCATACACACA-3' (annealing temperature: 53°C, 242 bp product). Primer sequences for the methylated *CDH1* gene were (sense) 5'-TTAGGTTAGAGGGTTATCGCGT-3' and (antisense) 5'-TAACTAAAAATTCACCTACCGAC-3' (annealing temperature: 57°C, 116 bp product), and those for the unmethylated *CDH1* were (sense) 5'-TAATTTTAGGTTAGAGGGTTATTGT-3' and (antisense) 5'-CACAACCAATCAACAACACA-3' (annealing temperature: 55°C, 97 bp product). A pair of positive (CpG universally methylated DNA; Intergen) and negative controls (distilled water) accompanied every amplification reaction. PCR products were separated in a 2% agarose gel or a non-denaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under ultraviolet illumination. The bisulfite reaction and MSP for all samples were repeated to confirm methylation status. Also some methylated and unmethylated PCR products were randomly selected and purified from the gels 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) 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 Analysis of *CDH13* by qRT-PCR

Using qRT-PCR, *18S* rRNA expression was detected in all 52 pituitary adenomas and 5 normal tissues. The median *CDH13* mRNA expression level (*CDH13/18S* rRNA) was 1.2 (range, 1.1–1.3) in normal pituitary tissues. We arbitrarily classified expression levels of less than one-half of this value, that is, *CDH13*/18S rRNA < 0.6, as significant reduction. Loss and significant reduction of *CDH13* expression were found in 6 and 22 pituitary

Table 2 Expression of *CDH13* mRNA in pituitary adenomas by quantitative real-time RT-PCR

	No.	*CDH13 mRNA expression*			P
	52	−	$\pm^a$	+	
Invasive	23	3 (13%)	14 (61%)	6 (26%)	< 0.05
Noninvasive	29	3 (10%)	8 (28%)	18 (62%)	

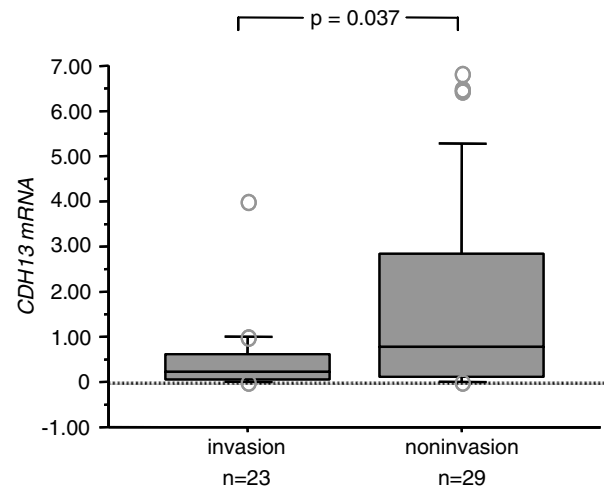[a]$\pm$, significantly lower expression of H-cadherin mRNA.

Figure 1 Results of quantitative real-time RT-PCR. *CDH13* mRNA expression in the invasion group is significantly lower than those in the non-invasion group ($P = 0.037$).

adenomas, respectively (Table 2). Reduced expression of *CDH13* was detected more frequently in invasive adenomas than in non-invasive adenomas (74 *vs* 38%, $P < 0.05$; Table 2) and *CDH13* mRNA expression was significantly lower in invasive adenomas than in non-invasive adenomas ($P = 0.037$; Figure 1).

Expression Analysis of E-Cadherin

In five normal adenohypophyseal samples, E-cadherin was expressed strongly on cell–cell boundaries of almost all hormone-producing cells, without cytoplasmic and nuclear localization. In pituitary adenomas, positive immunostaining of E-cadherin always showed a membranous pattern of reactivity without cytoplasmic and nuclear localization. The immunostaining results are illustrated in Figure 2.

E-cadherin expression was lost in 30% (21 of 69) tumors, significantly reduced in 32% (22 of 69; including 1 +, 6; 2 +, 8; and 3 +, 8) tumors and slightly reduced or normal in 38% (26 of 69; including 4 +, 17; 5 +, 7; and 6 +, 2) tumors. The expression of E-cadherin was significantly lower in invasive (grades IV and III) and macro- (grade II) than in non-invasive micro- (grade I) tumors ($P = 0.01$, $P = 0.029$, and $P = 0.015$, respectively;
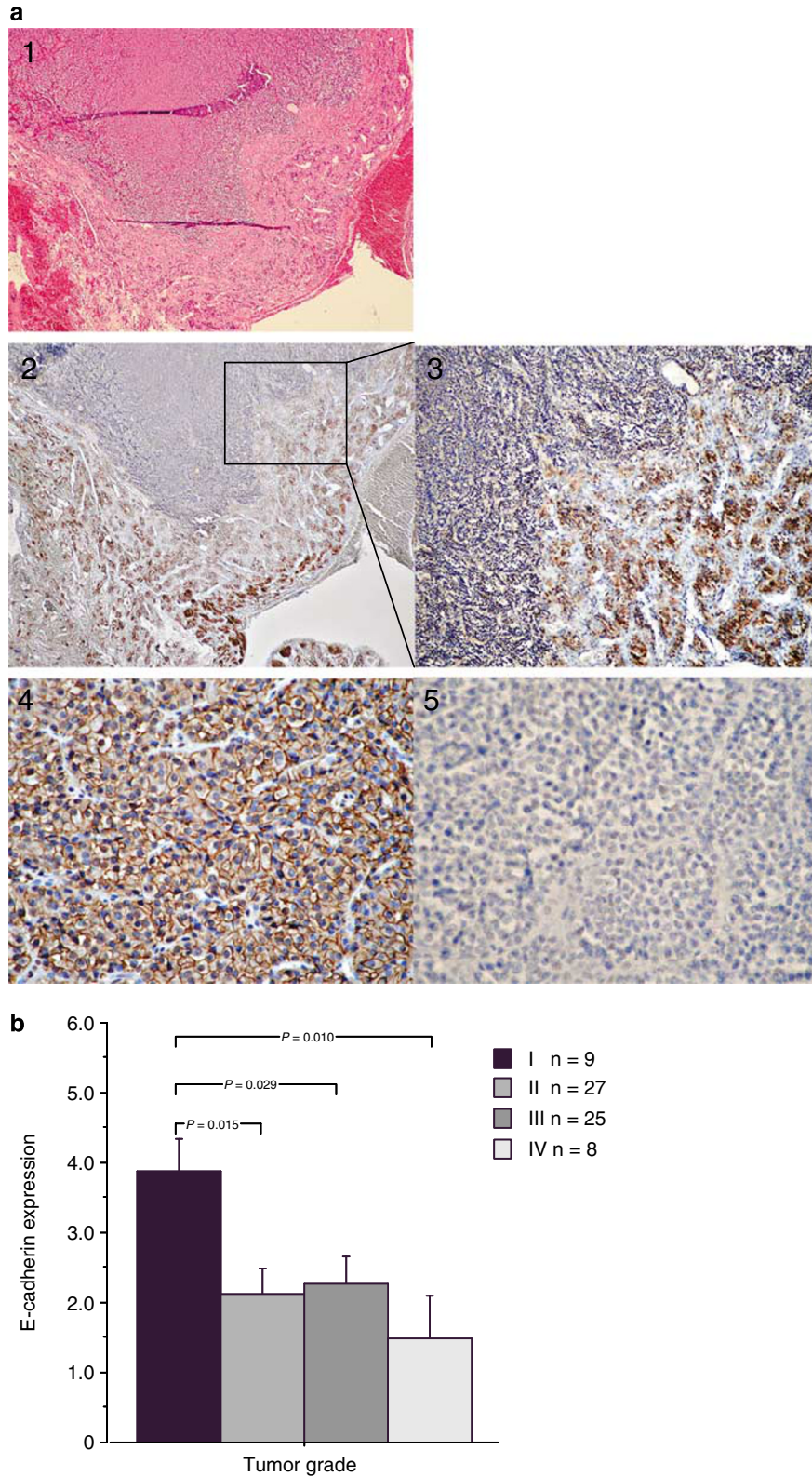
Figure 2 Analysis of E-cadherin expression level in pituitary tumors. (**a**) 1, 2, and 3, a pituitary adenoma does not have staining for E-cadherin, whereas the majority of surrounding non-tumorous cells have strong membranous positivity. 4, in a grade I case, tumor cells show strong membranous staining of E-cadherin. 5, in a grade IV case, E-cadherin immunoreactivity is not detected in tumor cells. (**b**) The expression of E-cadherin is significantly lower in invasive (grades IV and III) and macro- (grade II) than in non-invasive micro- (grade I) tumors ($P = 0.01$, $P = 0.029$, and $P = 0.015$, respectively).

Figure 2b). However, there was no significant difference among grades IV, III, and II tumors.

In addition, lost or downregulated E-cadherin expression was found in all of 8 GH cell adenomas with prominent fibrous bodies (0, 4 and ≤3 + , 4), but was just detected in 1 of 16 GH cell adenomas without fibrous bodies. Moreover, in prolactinomas, the expression of E-cadherin was lower in male patients than in females; however, the number of cases was small in this study.

Methylation Status of *CDH13* and *CDH1* in Pituitary Tumors

We used MSP to investigate the promoter methylation of *CDH13* and *CDH1* in 5 normal pituitary tissues and 69 pituitary adenomas. Representative examples are illustrated in Figure 3. We then analyzed the relationship between *CDH13* and *CDH1* methylation status and the clinicopathological characteristics of the 69 patients. The results are summarized in Table 1. Hypermethylation of the promoter region of *CDH13* and *CDH1* was detected in 30% (21 of 69) and 36% (25 of 69) of pituitary adenomas, respectively. However, there was no
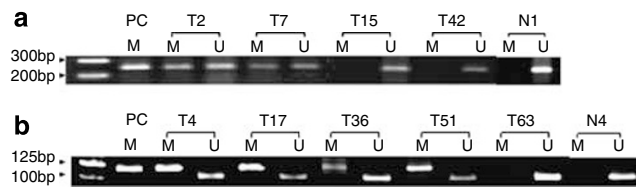
Figure 3 Representative MSP of *CDH13* and *CDH1* promoter methylation analysis in pituitary tumors and normal pituitary tissues. In each case, universally methylated genomic DNA was used as positive control for methylated allele. (**a**) *CDH13* promoter methylation was present in tumors 2 and 7. (**b**) *CDH1* promoter methylation was present in tumors 4, 17, 36, and 51. The methylated alleles of both genes were not detected in normal pituitary tissues.

methylation of either promoter in five normal pituitary tissues, suggesting that *CDH13* and *CDH1* promoter hypermethylation is tumor specific. Methylated patterns of *CDH13* and *CDH1* were found in all major types of pituitary adenomas: 25 and 21% of somatotroph adenomas, 33 and 50% of lactotroph adenomas, 25 and 25% of functioning corticotroph adenomas, 50 and 17% of silent corticotroph adenomas, and 14 and 50% of gonadotroph adenomas, respectively. The difference in the frequency of methylation between functional tumors (*CDH13*, 31%; *CDH1*, 31%) and non-functional tumors (*CDH13*, 29%; *CDH1*, 46%) was not statistically significant.

The methylation of *CDH13* was observed more frequently in invasive adenomas (42%) than in non-invasive adenomas (19%) ($P<0.05$; Table 3). The methylation of *CDH1* was more frequent in aggressive grade IV cases compared with grade I cases ($P<0.05$; Table 3). The methylation of either *CDH13* or *CDH1* was identified in 35 cases (51%) and was more frequently in grade IV or in invasive than in grade I or in non-invasive adenomas ($P<0.05$ and $P<0.05$, respectively; Table 4).

In addition, the specificity of MSP was confirmed by direct sequencing. In unmethylated MSP products, all cytosine nucleotides including those in the CpG islands changed to thymidines as a result of bisulfite modification. However, in methylated MSP products, cytosine nucleotides in the most CpG islands were unchanged (data not shown).

Correlation between Promoter Hypermethylation and Loss or Significant Reduction of Cadherin Expression

Five normal pituitary tissues with unmethylated alleles of *CDH13* and *CDH1* showed high levels of *CDH13* mRNA expression and strong expression of E-cadherin protein.

In 21 of 36 (58%) unmethylated tumors, *CDH13* mRNA levels were within the normal range. In

Table 3 *CDH13* and *CDH1* methylation in pituitary adenomas

Variable	No.	CDH13 methylation		P	CDH 1 methylation		P
	69	+	−		+	−	
Grade							
I	9	1 (11%)	8 (89%)		1 (11%)	8 (89%)	
II	27	6 (22%)	21 (78%)	0.4[a]	10 (37%)	17 (63%)	0.1[a]
III	25	10 (40%)	15 (60%)	0.1[b]	9 (36%)	16 (64%)	0.1[b]
IV	8	4 (50%)	4 (50%)	0.08[c]	5 (63%)	3 (37%)	<0.05[c]
Invasion				<0.05			0.31
Invasive	33	14 (42%)	19 (58%)		14 (42%)	19 (58%)	
Noninvasive	36	7 (19%)	29 (81%)		11 (31%)	25 (69%)	

[a]II *vs* I.
[b]III *vs* I.
[c]IV *vs* I.

contrast, *CDH13* mRNA was not detected or significantly reduced in 13 of 16 (81%) tumors with methylated *CDH13* promoters. There was a significant correlation between hypermethylation of the *CDH13* promoter and abnormal expression of *CDH13* ($P < 0.005$; Table 5). Similarly, E-cadherin expression was lost or significantly reduced in 22 of 25 (88%) methylated tumors. On the other hand, 23 of 44 (52%) unmethylated tumors showed strong E-cadherin expression. *CDH1* promoter hypermethylation was significantly correlated with loss or downregulation of E-cadherin protein expression ($P < 0.0005$; Table 5). However, these correlations were not completely consistent (Table 5). These findings suggested that silencing of the *CDH13* and *CDH1* genes can be caused not only by promoter methylation but also by other inactivating mechanisms. Unmethylated bands were found in almost all tumor tissues examined. These unmethylated alleles may be due to contaminated non-tumorous cells or tumor cells without epigenetic change and hemimethylated tumor cells.

Table 4 Analysis of combined *CDH13* and *CDH1* methylation in pituitary adenomas

Variable	No.	M^+M^+	M^+	M^-	P
Grade					
I	9	0	2 (22%)	7 (78%)	
II	27	4 (15%)	8 (30%)	15 (55%)	0.3[a]
III	25	5 (20%)	9 (36%)	11 (44%)	0.1[b]
IV	8	2 (25%)	5 (63%)	1 (12%)	<0.05[c]
Invasion					<0.05[d]
Invasive	33	7 (21%)	14 (43%)	12 (36%)	
Noninvasive	36	4 (11%)	10 (28%)	22 (61%)	
Total	69	11 (16%)	24 (35%)	34 (49%)	

M^+M^+, both *CDH13* and *CDH1* methylated alleles were detected; M^+, either *CDH13* or *CDH1* methylated alleles were detected; M^-, both *CDH13* and *CDH1* methylated alleles were not detected.
[a]II *vs* I.
[b]III *vs* I.
[c]IV *vs* I.
[d]$M^+M^+ + M^+$ *vs* M^-.

Discussion

Cell adhesion molecules, initially believed to account merely for the mechanical stability of cell–cell interactions, are now known to participate in most fundamental cell activities including proliferation, differentiation, mitogenesis, and apoptosis.[24] Changes in cell–cell and cell–matrix adhesion accompany the transition from benign tumor to invasive, malignant cancer and the subsequent metastatic dissemination of tumor cells.[24] Aberrant expression of H-cadherin and E-cadherin caused by *CDH13* and *CDH1* promoter hypermethylation has been reported in a variety of tumors and their effect on tumorigenesis and invasiveness has been elucidated.[3,4,11] In the current study, decreased mRNA expression of *CDH13* was detected in 54% (28/52) of pituitary tumor samples; E-cadherin protein expression was lost in 21 (30%) tumors and significantly reduced in 22 (32%) tumors. In addition, tumor-specific hypermethylation of *CDH13* and *CDH1* was detected in 30 and 36% of pituitary adenomas including all major types and in all tumor stages, respectively. Moreover, loss and downregulated expression of *CDH13* mRNA and E-cadherin protein were significantly associated with hypermethylation of *CDH13* and *CDH1*, respectively. Thus, our data, along with previous studies,[15–20] suggest that epigenetic alterations are common hallmarks of pituitary tumorigenesis. The aberrant expression of H-cadherin and E-cadherin and their DNA promoter hypermethylation may play an important role in pituitary tumor pathogenesis.

Invasiveness is regarded as one of the most salient determinants of surgical curability because it can limit surgical resection and lead to tumor regrowth.[25] Invasive adenomas are also believed to represent a biologically intermediate stage in tumor progression along the continuum from benign adenomas to carcinomas.[25] Understanding the biological basis of tumor invasion is crucial for developing new adjuvant treatment and enhancing the outcome of patients with aggressive pituitary tumors. Recent studies have described overexpression of two proto-oncogenes, pituitary tumor trans-

Table 5 Correlation of hypermethylation of *CDH13* and *CDH1* and expression of *CDH13* mRNA and E-cadherin protein in pituitary adenomas

CDH13	No.	*CDH13 methylation*		P	*E-cadherin*	No.	*CDH1 methylation*		P
Qrt-PCR[a]	52	+	−		*IHC*[b]	69	+	−	
−	6	5 (83%)	1	<0.005	—	21	14 (67%)	7	<0.0005
±[c]	22	8 (36%)	14		≤ 3[d]	22	8 (36%)	14	
+	24	3 (12.5%)	21		≥ 4	26	3 (12%)	23	

[a]Quantitative real-time RT-PCR.
[b]IHC, immunohistochemical analysis.
[c]±, significantly lower expression of H-cadherin.
[d]≤ 3, significantly lower expression of E-cadherin.

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forming gene (PTTG) and pituitary tumor-derived fibroblast growth factor receptor 4 (ptd-FGFR4), and matrix metalloproteinase-9, as associated with aggressive behavior in pituitary tumors.[26–28]

Because cell discohesiveness and detachment are important for tumor invasiveness, loss or down-regulated expression of H-cadherin and E-cadherin may facilitate tumor invasion.[2,29,30] Furthermore, E-cadherin also has a growth suppressor function by inducing cell-cycle arrest via upregulation of the cyclin-dependent kinase inhibitor p27.[31]

In this study, reduced expression of the *CDH13* gene was also associated with tumor aggressiveness (84% invasive cases *vs* 38% non-invasive cases in qRT-PCR analysis). Moreover, methylation of *CDH13* was observed more frequently in invasive adenomas (42%) than in non-invasive adenomas (19%) and was associated with high grade (grade IV (50%) *vs* grade I (11%)). Loss or significantly reduced E-cadherin expression was a frequent event (62%) in pituitary adenomas of all major types and correlated with tumor size and invasion. Methylation of *CDH1* was frequently found in more aggressive grade IV cases compared with grade I cases. These findings suggest that downregulated H-cadherin and E-cadherin expression and *CDH13* and *CDH1* gene hypermethylation may relate to invasive behavior in pituitary adenomas. E-cadherin may also play important roles in tumor proliferation.

Interestingly, promoter methylation of both *CDH13* and *CDH1* genes was detected in 11 cases, all of them representing large invasive tumors of high stage (grades II, III, and IV). Such cases were most frequent among grade IV or invasive tumors than in other grades or in non-invasive adenomas. Our findings suggest that concomitant methylation of *CDH13* and *CDH1* genes may be involved in pituitary tumor progression, as is the case in bladder, lung, gallbladder, and colorectal carcinoma.[6,32–34]

Many studies have shown that hypermethylation is a common mechanism of silencing the *CDH13* and *CDH1* genes. To address more fully these correlations, we have tried to quantitate the methylated alleles and determine the density of methylated CpG molecules in these genes using combined bisulfite restriction analysis (COBRA) and bisulfite sequencing[20] (data not shown). Unexpectedly, we detected hypermethylation pattern of *CDH13* and *CDH1* by COBRA and bisulfite sequencing only in a few samples, which had a hypermethylation pattern detected by MSP. This discrepancy resulting from different methods has been reported in other tumors and has been widely discussed.[35,36] MSP can detect 1 methylated allele in 1000 unmethylated alleles.[23] In contrast, using the bisulfite sequencing method, low numbers of methylated alleles ($<25\%$) may be missed.[23] However, our data indicate that hypermethylation of *CDH13* and *CDH1* gene promoter represents at least one mechanism that results in downregulation of E-cadherin and H-cadherin in

pituitary adenomas. Other mechanisms may be implicated as well. Loss of heterozygosity at chromosome 16q24.2–3 can cause downregulation of *CDH13* gene expression.[2] Mutation of the *CDH1* gene can lead to the expression of a non-functional protein.[11] The binding of the transcription factor Snail and/or Slug to E2 boxes in the *CDH1* promoter results in gene silencing.[37] Dysadherin interferes with E-cadherin function by downregulation of protein levels without affecting mRNA levels.[38] The stimulation of epidermal growth factor receptor also has been proposed for E-cadherin downregulation.[39] Future investigations are required to identify whether these mechanisms are active in pituitary adenomas.

In conclusion, we demonstrate decreased expression of H-cadherin and E-cadherin in about half of pituitary adenomas and loss of these adhesion molecules is associated with tumor aggressiveness. The methylation status of *CDH13* and *CDH1*, alone or in combination, is also related to invasive tumor behavior. Decreased expression of *CDH13* and *CDH1* is associated with aberrant methylation, but other mechanisms also may be implicated in downregulation of gene expression. Our results strongly suggest that silencing of the *CDH13* and *CDH1* genes may play an important role in the pathogenesis of pituitary adenomas and may be reliable predictor of tumor aggressiveness. Future studies will be required to identify the biological effects of silencing of these genes in pituitary tumorigenesis and invasion.

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