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Overexpression of HMGA2 relates to reduction of the *let-7* and its relationship to clinicopathological features in pituitary adenomas

Zhi Rong Qian[1], Sylvia L Asa[2], Haruhiko Siomi[3], Mikiko C Siomi[3], Katsuhiko Yoshimoto[4], Shozo Yamada[5], Elaine Lu Wang[1], Md Mustafizur Rahman[1], Hiroshi Inoue[6], Mitsuo Itakura[6], Eiji Kudo[1] and Toshiaki Sano[1]

[1]*Department of Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School of Medicine, Tokushima, Japan;* [2]*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada;* [3]*Division of Gene Function Analysis, Institute for Genome Research, The University of Tokushima, Tokushima, Japan;* [4]*Department of Medical Pharmacology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan;* [5]*Department of Neurosurgery, Toranomon Hospital, Tokyo, Japan and* [6]*Division of Genetic Information, Institute for Genome Research, The University of Tokushima, Tokushima, Japan*

High-mobility group A2 is highly expressed during embryogenesis and in various benign and malignant tumors. Recent studies report that high-mobility group A2 is negatively regulated by the *let-7* microRNAs (miRNAs) family *in vitro*. The development of pituitary adenomas in high-mobility group A2 transgenic mice showed that high-mobility group A2 may be involved in pituitary tumorigenesis. However, no studies have investigated the clinical significance of high-mobility group A2 and its relationship to the *let-7* miRNA family in human pituitary adenomas. Using immunohistochemistry, we analyzed high-mobility group A2 expression with respect to various clinicopathologic factors in 98 pituitary adenomas. Overexpression of high-mobility group A2 was observed in 39% (38/98) of pituitary adenomas compared with normal adenohypophysial tissue and was frequently found in adenomas including prolactin (PRL), adrenocorticotrophic hormone, or follicle-stimulating hormone/luteinizing hormone and in null cell adenomas, but relatively rare in growth hormone (GH) and mixed GH/PRL adenomas. High-mobility group A2 expression was significantly associated with tumor invasion ($P<0.05$) and was significantly higher in grade IV than in grades I, II, and III adenomas ($P<0.05$). High levels of high-mobility group A2 expression were more frequently observed in macroadenomas than in microadenomas ($P<0.05$). High levels of high-mobility group A2 expression also significantly correlated with the proliferation marker Ki-67 ($P<0.0001$). Real-time quantitative RT-PCR analysis was carried out to evaluate the expression of *let-7* in 55 pituitary adenomas. Subsequently, decreased expression of *let-7* was confirmed in 23 of 55 (42%) adenomas and was correlated with high-grade tumors ($P<0.05$). An inverse correlation between *let-7* and high-mobility group A2 expression was evident ($R=-0.33$, $P<0.05$). These findings support a causal link between *let-7* and high-mobility group A2 whereby loss of *let-7* expression induces high-mobility group A2 upregulation that represents an important mechanism in pituitary tumorigenesis and progression.
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Correspondence: Dr ZR Qian, MD, PhD and Dr T Sano, MD, PhD, Department of Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School of Medicine, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan.
E-mails: zrqian@basic.med.tokushima-u.ac.jp and sano@basic.med.tokushima-u.ac.jp
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Pituitary adenomas are common intracranial neoplasms, comprising 10–15% of diagnosed brain tumors.[1] They may arise from any of the five differentiated cell types within this gland: somatotropes, lactotropes, corticotropes, thyrotropes, and gonadotropes, which secrete growth hormone (GH), prolactin (PRL), adrenocorticotrophic hormone (ACTH), thyroid-stimulating hormone (TSH), and gonadotropins (follicle-stimulating hormone (FSH)

and luteinizing hormone (LH)), respectively. Pituitary adenomas are usually biologically benign, but often associated with specific endocrine syndromes, such as acromegaly, amenorrea–galactorrea, Cushing disease, TSH-induced hyperthyroidism, and hypopituitarism. The tumors can also cause tumor mass effect (ie, local compressive effects on brain structures and cranial nerves). The identification of molecular pathways leading to pituitary tumorigenesis represents one of the major challenges in endocrine oncology. Several factors, such as gene mutations, hypothalamic dysregulation, and locally produced growth factors, appear to be, though controversial, involved in the transformation of pituitary cells.[2]

The high-mobility group A (HMGA) proteins are nonhistone chromosomal proteins that bind through their AT-binding motifs to the minor groove of AT-rich DNA strands. They have no intrinsic transcriptional activity but can modulate transcription by altering chromatin architecture.[3–5] High-mobility group A2 (HMGA2) proteins are widely expressed during embryogenesis, whereas their expression is low or absent in normal adult tissues.[6,7] HMGA2 proteins are involved in many diverse biological processes such as regulation of transcription, embryogenesis, differentiation, neoplastic transformation, and integration and expression of viral genomes.[8] HMGA2 overexpression is a hallmark of various benign and malignant tumors and is also associated with a highly malignant phenotype and is a poor prognostic index.[9] It was reported that transgenic mice overexpressing HMGA2 develop pituitary adenomas secreting PRL and GH and may be involved in pituitary tumorigenesis.[10] However, HMGA2 expression was just studied in a small series of human sporadic prolactinomas and nonfunctional pituitary adenomas.[11,12] To date there has been no study of HMGA2 expression in a large series of human pituitary adenomas including all subtypes and its relationship to clinicopathological features in these tumors.

MicroRNAs (miRNAs) represent an emerging class of small endogenous noncoding RNAs (approximately 18–24 nucleotides) and they may regulate gene expression at the posttranscription level by direct cleavage of a target mRNA using interference machinery (mRNA cleavage) or by inhibition of protein synthesis.[13,14] miRNAs have been implicated in a variety of developmental and physiological processes, including cell proliferation and differentiation, apoptosis, metabolism, and morphogenesis.[15] Recent evidence has shown that miRNA misexpression correlates with various human cancers and indicates that some miRNAs can function as oncogenes or tumor suppressors.[14] *Let-7* was first identified in *Caenorhabditis elegans* and is highly conserved in *C. elegans*, *Drosophila*, zebrafish, and humans.[16–18] *Let-7* is barely detectable in embryonic stages but increases in differentiated and mature tissues.[18] *Let-7* was indicated as a tumor suppressor in lung and colon cancer through targeting of *RAS* oncogene.[19] Recently, several studies reported that *let-7* can negatively regulate HMGA2 expression in a mouse model system,[20] in head and neck cancers,[21] in uterine leiomyomas,[22] in lung cancer cell lines,[23] and in ovarian cancers.[24] However, very few data are available concerning the role of miRNA in pituitary physiology and disease and there have been no reports investigating a possible correlation between HMGA2 and the *let-7* miRNA in pituitary adenomas.

In this study, we investigated the expression of HMGA2 in a series of 98 human pituitary adenomas of the various types and in 4 normal pituitary glands and compared these data with tumor type, size, invasiveness, and the labeling index of the proliferation marker Ki-67 antigen. We also investigated possible associations between HMGA2 expression and *let-7* miRNA expression in pituitary adenomas.

Materials and methods

Human Normal and Pituitary Adenoma Samples

Normal human adenohypophyses were obtained at autopsy from four patients without endocrine dysfunction at Tokushima University Hospital (Tokushima, Japan). These tissues were examined using the hematoxylin–eosin stain and immunocytochemistry to exclude the possibility of incidental tumors. Ninety-eight postsurgical pituitary adenoma tissue samples were obtained from Tokushima University Hospital and Toranomon Hospital (Tokyo, Japan). These included 28 somatotroph, 5 mammosomatotroph, 16 lactotroph, 18 corticotroph (8 associated with Cushing's disease and 10 silent), 3 thyrotroph, 22 gonadotroph, 3 silent subtype-3, and 3 null cell adenomas (Table 1). Tumor size and invasiveness were defined on the basis of preoperative radiological investigations and operative findings, with a modified Hardy's classification.[25] Grade I (microadenomas, <1 cm in diameter) and grade II (enclosed macroadenomas with or without suprasellar extension, ≥ 1 cm in diameter) tumors were defined as noninvasive. Grade III (local invasion of sphenoid and/or cavernous sinus) and grade IV tumors (with central nervous system/extracranial spread with or without metastasis) were considered to be invasive. Thus, 98 tumors included 11 tumors of grade I, 43 tumors of grade II, 32 tumors of grade III, and 12 tumors of grade IV (54 noninvasive and 44 invasive adenomas, Tables 1 and 3). None of the tumors examined in this study had evidence of postoperative recurrence.

Immunohistochemical

To detect HMGA2 protein and Ki-67 antigen, immunolocalization experiments were carried out

Table 1 Clinical and pathological characteristics of 98 pituitary adenomas used for immunohistochemistry

	Case numbers	*Tumor size*		*Invasiveness*	
		Macroadenoma	*Microadenoma*	*Invasive*	*Noninvasive*
Patients (*n*, %)	98	87 (89)	11 (11)	44 (45)	54 (55)
*Gender (*n, *%)*					
Male	46	42 (91)	4 (9)	18 (39)	28 (61)
Female	52	45 (87)	7 (13)	26 (50)	26 (50)
*Tumor type (*n, *%)*					
GH (acromegaly)	28	22 (79)	6 (21)	10 (36)	18 (64)
GH/PRL (acromegaly)	5	5 (100)	0	2 (40)	3 (60)
PRL (prolactinoma)	16	13 (81)	3 (19)	9 (56)	7 (43)
ACTH (Cushing)	8	7 (88)	1 (12)	2 (25)	6 (75)
ACTH (silent)	10	9 (90)	1 (10)	5 (50)	5 (50)
FSH/LH (NF)	22	22 (100)	0	9 (41)	13 (59)
Null cell (NF)	3	3 (100)	0	3 (100)	0
TSH	3	3 (100)	0	1 (33)	2 (67)
Silent subtype 3	3	3 (100)	0	3 (100)	0
Total (*n*, %)	98	87 (89)	11 (11)	44 (45)	54 (55)

GH, GH cell adenoma; GH/PRL, mixed GH cell–PRL cell adenoma; PRL, PRL cell adenoma; ACTH, ACTH cell adenoma; FSH/LH, FSH/LH cell adenoma; Null cell, null cell adenoma; NF, nonfunctional; TSH, TSH cell adenoma; Silent subtype 3, silent subtype 3 adenoma.

on sections from representative blocks of paraffin-embedded tissues using the labeled streptavidin–biotin method, as previously described.[26,27] After deparaffinization and antigen retrieval using an autoclave oven technique, sections were incubated at 4°C overnight with goat polyclonal anti-HMGA2 antibody (1:50; HMGI-C, S-15, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with Ki-67 antigen mouse monoclonal antibody (1:100; DakoCytomation, Glostrup, Denmark). Antigen–antibody complexes were detected using the cobalt-3,3′-diaminobenzidine reaction. The slides were counterstained lightly with hematoxylin or 1% methyl green and mounted for microscopic examination. A squamous cell carcinoma known to be positive for HMGA2 was used as a positive control. Sections incubated in PBS without the primary antibody served as negative controls.

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 nuclear staining was considered to present a positive stain for HMGA2 and Ki-67. A total of 500–1000 cells were counted and the percentage of HMGA2-stained tumor cells was scored on a scale of 0–4 (0, no staining; 1+, 1–20%; 2+, 20–50%; 3+, 50–80%; 4+, >80%). Furthermore the expression level of HMGA2 was divided as following three groups: negative, (0); moderate, (1+, 2+); high, (3+, 4+).

The Ki-67 antigen labeling index was determined by counting the number of positive cells in a total of 500–1000 tumor cells observed in several representative high-power fields (×400). The results were expressed as percentage of tumor cells with positive nuclei.

RNA Isolation and Real-Time Quantitative RT-PCR Analysis

Total RNA isolation and enrichment of small RNAs were performed using a *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. For the miRNA quantitative RT-PCR (qRT-PCR) analysis, enriched small RNAs from 4 normal human adenohypophysis and 55 human pituitary adenomas were used. All samples were frozen and stored at −80°C. Because *let-7* family has similar function, we used a primer set that may amplify multiple *let-7* family members. The expression of human mature *let-7* (*hsa-let-7*) miRNA was analyzed using a *mir*Vana qRT-PCR miRNA detection kit (Ambion catalog nos. AM1558 and AM30000) according to manufacturer's protocol. Ubiquitously expressed *U6* small nuclear RNA (Ambion catalog no. AM30303) was used for normalization. HeLa cell was chosen as positive control for *let-7* expression. Briefly, reverse transcription reaction was performed with 20 ng of total RNA using gene-specific RT primers for *let-7* and *U6*, respectively. Real-time PCR was performed on the 7900HT Fast Real-Time PCR System (Applied Biosystems), in a 25 μl reaction mixture consisting of 10 μl reverse transcription product, 5 μl *mir*Vana 5× Buffer, 0.5 μl 50× ROX, 0.5 μl *mir*Vana PCR primers and thermostable DNA polymerase (1 unit). PCR cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was analyzed in duplicate. Serial dilutions of cDNA from a pool of three normal pituitaries were used for the standard curve calculation. The standard curve method was used to determine

relative quantitation of *let-7*. The relative amount of *let-7* in each sample was normalized to the *U6 RNA*. The fold change values indicate the relative change in the expression levels between tumors samples and normal pituitary tissues assuming that the value of normal sample was equal to 1. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was also be used to confirm the results from the standard curve method.[28,29]

Statistical Analyses

To determine the significance of associations between different variables, data were statistically analyzed by Mann–Whitney *U*-test, Kruskal–Wallis test, χ^2-test, Spearman's correlation coefficient, and Pearson's correlation coefficient, using StatView J-4.5 software (Abacus Concepts, Berkeley, CA, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

Overexpression of HMGA2 Oncoprotein in Human Pituitary Adenomas

In this study, we examined HMGA2 expression in four normal pituitary glands and the 98 adenoma specimens. There was no appreciable immunostaining of HMGA2 in all normal pituitary tissues tested (Figure 1a). A clear nuclear staining for HMGA2, without membrane or cytoplasmic localization, was detected in 38 (39%) pituitary adenomas (Figure 1b–d; Table 2). HMGA2 immunoreactivity was frequently observed in three subtypes of pituitary adenomas; FSH/LH cell adenomas (15 of 22 cases, 68%), ACTH cell adenomas (12 of 18 cases, 67%), and PRL cell adenomas (5 of 16 cases, 31%). However, HMGA2 immunoreactivity was found rarely in GH cell adenomas (2 of 26 cases, 7%) and was not found in mixed GH/PRL adenomas ($P<0.0001$, Table 2).

Expression of HMGA2 as high level and moderate level was detected in 24 of 98 and 14 of 98 adenomas, respectively. Of 98, 60 adenomas showed negative expression of HMGA2 (Table 3). The expression of HMGA2 was significantly higher ($P<0.05$, Figure 1e) and tended to be present with greater frequency in invasive adenomas than in noninvasive adenomas (Table 3). In addition, the expression of HMGA2 was more frequent ($P<0.05$, Table 3) and tended to be higher in macroadenomas than in microadenomas (Figure 1f). The overall level of HMGA2 expression was significantly higher in grade IV adenomas than grades I, II, and III ($P<0.05$, Figure 1g). Also high-level expression of HMGA2 case was frequently detected in grade IV ($P<0.05$, Table 3). Furthermore, Ki-67 labeling index correlated with HMGA2 overexpression ($R=0.395$, $P<0.0001$) and progressively increased from HMGA2 negative expression cases to moderate expression cases and high expression cases ($P<0.0001$, Figure 1h). The overexpression of HMGA2 was not related to patient age and gender (data not shown).

Aberrant *Let-7* Expression in Human Pituitary Adenomas

Real-time qRT-PCR method was used to address the levels of *let-7* in 4 normal and 55 adenomatous pituitary samples. As shown in Figure 2a and Table 4, compared with normal tissues, the reduced expression of *let-7* was found in 23 (41.8%) adenomas (most cases exhibited >50% reduction). Slight upregulation of *let-7* expression levels was also observed in the 15 (27%) adenomas (Figure 2a; Table 4; upregulation rates were less than 3-fold, most cases showed 1 to 2-fold level expression), whereas high upregulation of *let-7* was detected in remaining 17 (31%) cases (upregulation rates were over 3-fold, few cases showed over 10-fold level expression). HeLa cells showed high level of *let-7* (upregulation rate, fourfold). These aberrant expressions of *let-7* may have tumor type–specificity. Reduction of *let-7* was frequently detected in three subtypes of pituitary adenomas: PRL cell adenomas (6 of 9, 68%), ACTH cell adenomas (7 of 12, 58%), and FSH/LH cell adenomas (6 of 17 cases, 35%). However, in GH cell adenomas, reduction of *let-7* was found only in 1 of 12 (8%) cases, whereas high upregulation was observed in 6 of 12 (50%) cases (Table 4).

When analyzed the clinicopathological characteristics of 23 pituitary adenomas with reduced *let-7* expression, there were no notable differences on patient age and sex (data not shown). Interestingly, the expression level of *let-7* was significantly lower in high-grade (III, IV) adenomas than in low-grade

Figure 1 Detection of HMGA2 immunoreactivity in pituitary normal tissues and 98 adenomas. (**a**) In normal pituitary cells HMGA2 showed negative immunoreaction. In contrast to normal cells, positive nuclear staining of HMGA2 was observed in tumor cells such as prolactinoma (3+; **b**), silent ACTH cell adenoma (4+; **c**) and FSH/LH cell adenoma (4+; **d**). The immunoreactivity of HMGA2 is variable but always very intense throughout the nuclei. The differences in immunostaining scores of HMGA2 between invasive and noninvasive adenomas or macroadenomas and microadenomas or among tumor grades were analyzed, respectively. (**e**) HMGA2 expression levels were significantly higher in invasive pituitary adenomas than in noninvasive pituitary adenomas ($P<0.05$, Mann–Whitney *U*-test). (**f**) HMGA2 expression levels were potentially higher in macroadenomas than in microadenomas ($P=0.36$, Mann–Whitney *U*-test). (**g**) HMGA2 expression levels were significantly correlated with tumor grade ($P<0.05$, Kruskal–Wallis test). (**h**) Ki-67 labeling index levels were significantly correlated with HMGA2 expression ($P<0.05$, Kruskal–Wallis test). Immunoreactivity score: 0, no staining; 1+, 1–20%; 2+, 20–50%; 3+, 50–80%; 4+, >80%. Negative, scale 0; moderate, scales 1+ and 2+; high, scales 3+ and 4+.

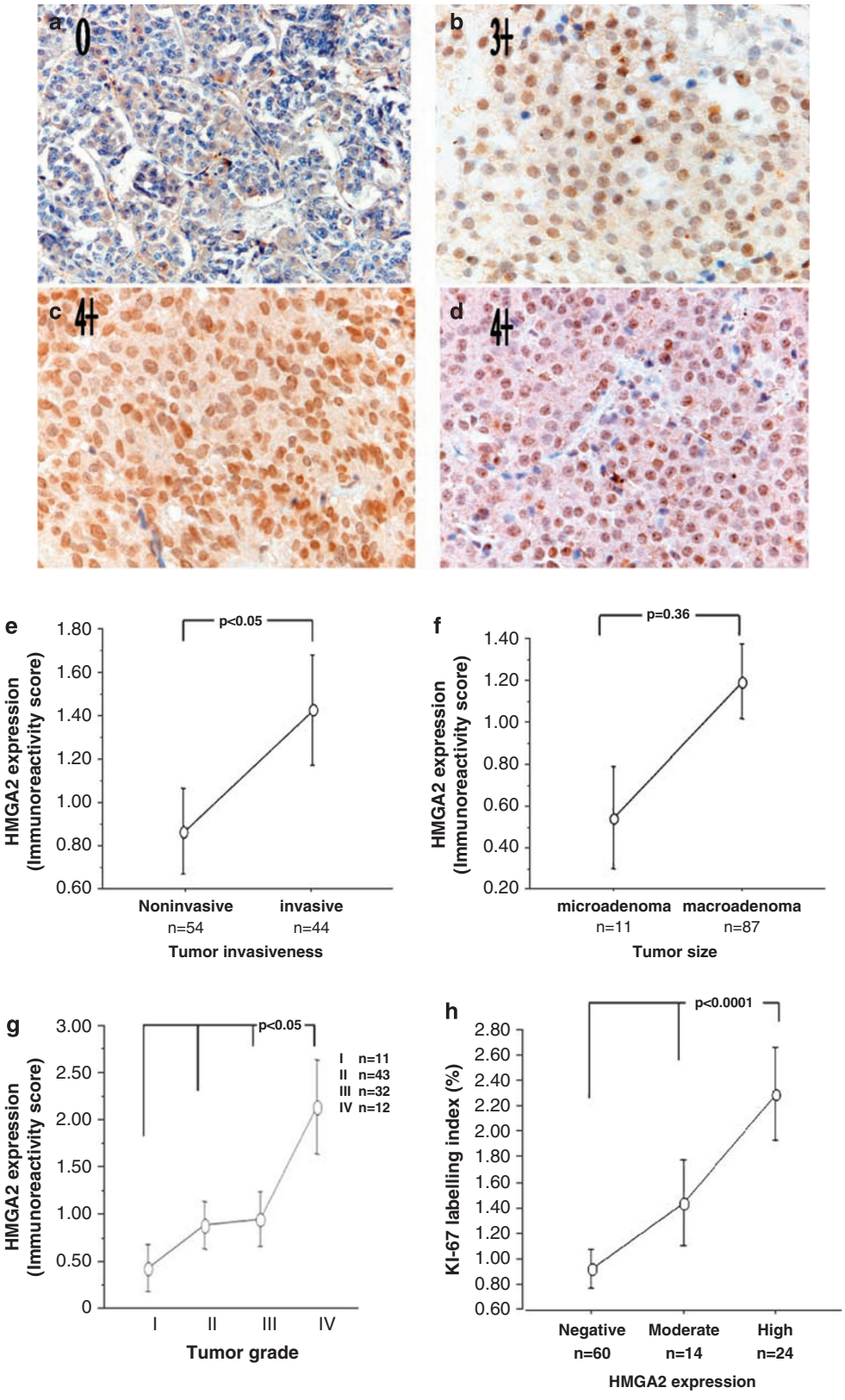
a
0
b
3+
c
4+
d
4+
e
p<0.05
HMGA2 expression (Immunoreactivity score)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
Noninvasive
n=54
invasive
n=44
Tumor invasiveness
f
p=0.36
HMGA2 expression (Immunoreactivity score)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
microadenoma
n=11
macroadenoma
n=87
Tumor size
g
p<0.05
HMGA2 expression (Immunoreactivity score)
3.00
2.50
2.00
1.50
1.00
0.50
0
I n=11
II n=43
III n=32
IV n=12
I
II
III
IV
Tumor grade
h
p<0.0001
KI-67 labelling index (%)
2.80
2.60
2.40
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
Negative
n=60
Moderate
n=14
High
n=24
HMGA2 expression

Table 2 The nuclear expression of HMGA2 protein in 98 pituitary adenomas

Pathological	*No. of cases*	*Immunoreactivity score of HMGA2*					*HMGA2⁺ (%)*	P
Tumor type		*0*	*1+*	*2+*	*3+*	*4+*	*(≥1+)*	
GH	28	26	0	2	0	0	2 (7)	<0.0001
GH/PRL	5	5	0	0	0	0	0	
PRL	16	11	1	2	2	0	5 (31)	
ACTH	18	6	4	0	2	6	12 (67)	
FSH/LH	22	7	1	3	3	8	15 (68)	
Null	3	0	0	0	0	3	3 (100)	
TSH	3	3	0	0	0	0	0	
Silent subtype 3	3	2	1	0	0	0	1 (33)	
Total	98	60	7	7	7	17	38 (39)	

HMGA2⁺, immunopositive for HMGA2; immunoreactivity score: 0, no staining; 1+, 1–20%; 2+, 20–50%; 3+, 50–80%; 4+, >80%. The χ^2-test was used to analyze the differences in frequencies of HMGA2 immunoreaction among each subtype of pituitary adenomas.

Table 3 Relationship of HMGA2 expression with tumor characteristics

Variable	*No. of cases*	*HMGA2 expression*			P
	98	*Negative (n = 60)*	*Moderate (n = 14)*	*High (n = 24)*	
Invasion					>0.05
Invasive	44	24 (54%)	6 (14%)	14 (32%)	
Noninvasive	54	36 (67%)	8 (15%)	10 (18%)	
Tumor size					<0.05
Micro	11	7 (64%)	4 (36%)	0	
Macro	87	53 (60%)	10 (12%)	24 (28%)	
Grade					<0.05
I	11	7 (64%)	4 (36%)	0	
II	43	29 (68%)	4 (9%)	10 (23%)	
III	32	21 (66%)	3 (9%)	8 (25%)	
IV	12	3 (25%)	3 (25%)	6 (50%)	

Negative, scale 0; moderate, scales 1+ and 2+; high, scales 3+ and 4+.
The χ^2-test was used to analyze the differences in frequencies of HMGA2 immunoreaction among each group of pituitary adenoma.

(I and II) adenomas (Figure 2b), and potentially lower in invasive adenomas than in noninvasive adenomas, although the differences were not statistically significant (data not shown).

Inverse Correlation between *Let-7* Expression and HMGA2 Expression

At first, the expression of HMGA2 was negative in all four normal pituitary tissues that showed abundant *let-7* by RT-PCR (data not shown). We further investigated the relationship between *let-7* expression and HMGA2 expression in 55 pituitary adenomas through several viewpoints. Generally, 25 of 55 adenomas showed HMGA2 immunoactivity (Figure 2a; Table 4). Pituitary adenomas with positive HMGA2 immunostaining were more frequently detected in *let-7* downregulated adenomas (14 of 23, 61%) than in *let-7* slightly upregulated adenomas (7 of 15, 47%) and *let-7* highly upregulated adenomas (4 of 17, 24%; $P<0.05$, Figure 2a; Table 4). Notably, high-level expression of HMGA2 was not detected in *let-7* highly upregulated adenomas (Table 4). In addition, HMGA2 expression levels were higher in *let-7* downregulated adenomas than in *let-7* slightly upregulated adenomas and *let-7* highly upregulated adenomas ($P<0.05$, Figure 3a). On the other hand, significantly lower expression of *let-7* was detected in pituitary adenomas with high HMGA2 expression ($P<0.05$, Figure 3b). Finally, this inverse correlation between the expression of *let-7* and HMGA2 in human pituitary adenomas was confirmed using Pearson's correlation coefficient analysis ($r=-0.33$, $P<0.05$, Figure 3c) and Spearman's correlation coefficient analysis ($P=0.003$).

Discussion

A high expression of HMGA2 has been detected in many kinds of benign tumor and cancer. It is also associated with a highly malignant phenotype and is a poor prognostic index.[9] As *HMGA2* transgenic

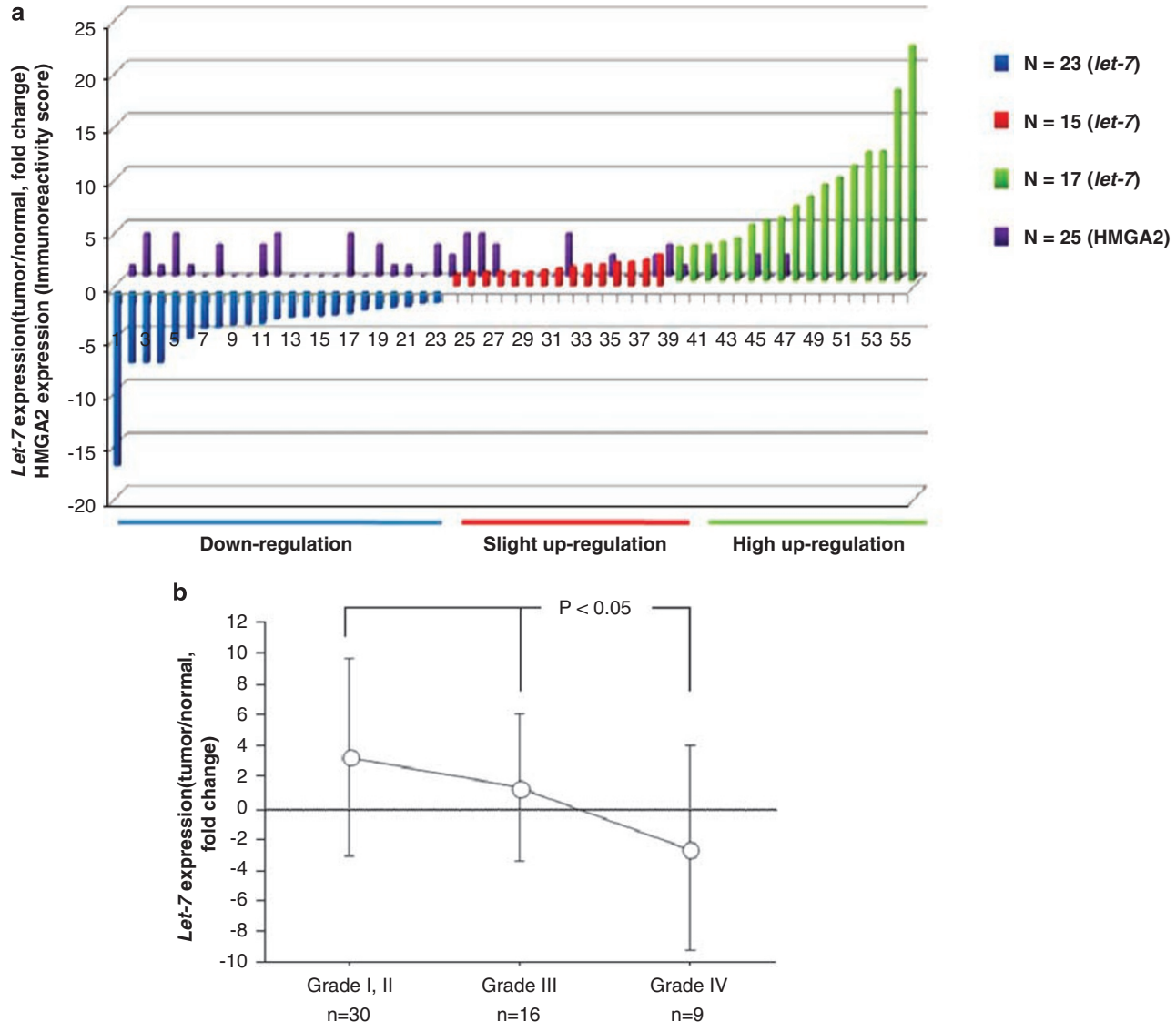

Figure 2 *Let-7* expression and HMGA2 immunostaining profile analyzed in 55 pituitary adenomas. (**a**) The value of *let-7* expression in normal pituitary tissues was equal to onefold. The reduced expression of *let-7* was found in 23 (41.8%) adenomas (blue square columns, downregulation rates were −1.10 to −16.41-fold, 17 cases showed over 2-fold change). Slight upregulation of *let-7* was also observed in the 15 (27.2%) adenomas (red square columns, upregulation rates were less than 3-fold, 1.06 to 2.98-fold, 9 cases showed 1 to 2-fold level expression), whereas high upregulation of *let-7* was detected in remaining 17 (31%) cases (green square columns, upregulation rates were over 3-fold, 3.24 to 22.21-fold, 5 cases showed over 10-fold level expression). In addition, 25 of 55 adenomas showed HMGA2 immunoactivity (purple square columns, in this figure, immunoreactivity score was converted to fold change temporarily: 0 = 0-fold; 1 + = 1-fold; 2 + = 2-fold; 3 + = 3-fold; 4 + = 4-fold). HMGA2 immunostaining were detected in 14 of 23 *let-7* downregulated adenomas, 7 of 15 in *let-7* slightly upregulated adenomas and in 4 of 23 *let-7* highly upregulated adenomas, respectively. (**b**) The reduction of *let-7* expression was potentially related to tumor grade. In grade IV, tumors showed the lowest level of *let-7* ($P<0.05$, Kruskal–Wallis test). Grade I and grade II adenomas were placed together because grade I has a small number of cases ($n=2$).

mice develop mixed GH-PRL cell pituitary adenomas,[10] and upregulation of HMGA2 protein has been found in human prolactinomas and nonfunctioning adenomas,[11,12] *HMGA2* is a reasonable candidate oncogene for involvement in pituitary tumorigenesis. To our knowledge, our study is the first report concerning the clinical significance of HMGA2 overexpression in pituitary adenomas. In this study, upregulated expression of HMGA2 protein was found in a significant fraction (39%) of pituitary adenomas and associated with prognostic factors such as tumor grade, extent of invasion, tumor size, and cell proliferation marker. A strong correlation existed between HMGA2 overexpression and tumor cell invasion has been detected in breast cancer and gastric cancer.[30,31] In oral squamous cell carcinomas, strong staining of HMGA2 and loss of E-cadherin expression were detected at the invasive front of tumor.[32] Our previous study also demonstrated that tumor-specific downregulation of E-cadherin and H-cadherin related to invasiveness of pituitary adenoma.[27] HMGA2 may be involved in tumor cell

Table 4 The expression of *let-7* and HMGA2 in 55 pituitary adenomas

Variable	*No. of cases*	*Let-7 expression (fold change)*			P
		−	+	++	
	55	*(n = 23)*	*(n = 15)*	*(n = 17)*	
GH	12	1 (8%)	5 (42%)	6 (50%)	
PRL	9	6 (67%)	0	3 (33%)	
ACTH	12	7 (58%)	2 (17%)	3 (25%)	
FSH/LH	17	6 (35%)	7 (41%)	4 (24%)	
TSH	2	1 (50%)	0	1 (50%)	
Silent subtype 3	3	2 (67%)	1 (33%)	0	
HMGA2					
Negative	30	9 (39%)	8 (54%)	13 (76%)	<0.05
Moderate	12	6 (26%)	2 (14%)	4 (24%)	
High	13	8 (35%)	5 (33%)	0	

−, downregulated levels; +, normal and slightly upregulated levels; ++, highly upregulated levels. The χ^2-test was used to analyze the differences in frequencies of *let-7* expression among each group of pituitary adenoma classed by HMGA2 immunoreaction.

invasion because of association with epithelial–mesenchymal transition that triggers tumor cell invasion. HMGA2 may regulate transcription factors, such as Snail, Slug, Twist, and inhibitor of differentiation 2, resulting in repressing E-cadherin expression and are contributed to a response to TGF-β that causes epithelial–mesenchymal transition.[33] In addition several studies have demonstrated that blocking of HMGA2 protein synthesis has a negative effect on tumor cell proliferation.[10,34] Cell growth and oncogenic activity of HMGA proteins are based on their ability to downregulate or upregulate the expression of genes such as *E2F1*, *cyclin A*, and *p53* that have a crucial function in the control of cell proliferation.[9] These observations supported our findings that HMGA2 overexpression is related to tumor development and invasiveness in pituitary adenomas.

Characterization of HMGA2 overexpression with clinicopathological data in a large series of pituitary adenomas including all major types were not reported. Specially, studies on expression of HMGA2 in GH cell and ACTH cell adenomas have not been reported. Interestingly, we observed preferable expression of the HMGA2 protein in either ACTH or FSH/LH cell adenomas, as compared to GH or mixed GH-PRL cell adenomas. This is inconsistent with the observations in *HMGA2* transgenic models developing mixed GH-PRL cell adenomas.[10] The reasons for this discrepancy were currently unknown, but the underlying oncogenic mechanisms of pituitary adenoma may vary depending on species and cell types. This tumor type-specific alteration also has been reported for some tumor suppressor genes, *CDKN2A*, *RB1*, and *RASSF1A*; and some oncogenes, *Gsp* and *FGFR4*.[28,35–38] These observations indicate that different types of pituitary adenomas may have distinct etiologic factors and pathogenetic mechanisms.

Let-7 is one of the founding members of the miRNA family, and several lines of evidence suggest that human *let-7* acts as a putative tumor suppressor. In human, various *let-7* genes map to chromosomal regions altered or deleted in human tumors.[39] Expression of *let-7* has been found to be downregulate in lung, colon, and ovarian cancers,[24,40,41] and its expression levels to be associated with cancer progression. In our study, at first, sequencing analysis of miRNA library implied that *let-7* levels were downregulated in pituitary adenomas (data not shown); then using qRT-PCR, we demonstrated that *let-7* expression was significantly decreased in more than one-third of the pituitary adenomas, an observation consistent with the previous microarray data from a small series of pituitary adenomas.[42] We further found that reduced *let-7* expression correlated with high tumor grade. These findings thus provide a clinical evidence supporting that *let-7* may function as a tumor suppressor miRNA, involving in the development and progression of human pituitary adenomas.

Recently, several groups independently reported that expression of *HMGA2* is suppressed by *let-7 in vitro*,[20,23] and that disrupting the *let-7* regulation of the HMGA2 enhances oncogenic transformation.[20] Intriguingly, human *HMGA2* gene has been known as a frequent target of chromosomal rearrangements that cause the loss of the C terminus of the protein and the 3′-UTR of the mRNA, the latter of which contains putative *let-7*-binding sites.[9] In prolactinomas, upregulation of HMGA2 may be associated with amplification and/or rearrangement of the *HMGA2* locus; but, in contrast to prolactinomas, rearrangement of the *HMGA2* locus was rare in nonfunctional pituitary adenomas with HMGA2 overexpression.[9] In our study, we confirmed that there was a significant inverse correlation between *let-7* and HMGA2 expressions in human pituitary adenomas. In addition to loss of *let-7*-mediated repression because of truncation of HMGA2, *let-7* reduction itself also may contribute to HMGA2 overexpression. Our findings first addressed the clinical significance of the *let-7*/HMGA2 connection in a large series of human pituitary tumors.

However, other aberrant expression of *let-7* also has been detected. Upregulation of *let-7* has been demonstrated in pituitary adenomas and some of them showed very high level of *let-7*. Except for functioning as tumor suppressor, does *let-7* have oncogenic function in pituitary tumorigenesis through repressing other targets? Oncogenic function of *let-7a-3* has been reported in a lung cancer model.[43] Furthermore, we could not always demonstrate the concerted expression changes of *let-7* and HMGA2 in pituitary adenomas. This could be because of technical/interpretive difficulties, but is more likely because of other possibilities. In some *let-7* downregulated adenomas, HMGA2 expression has not been observed. This phenomenon implied that reduced *let-7* may not be only one initial reason

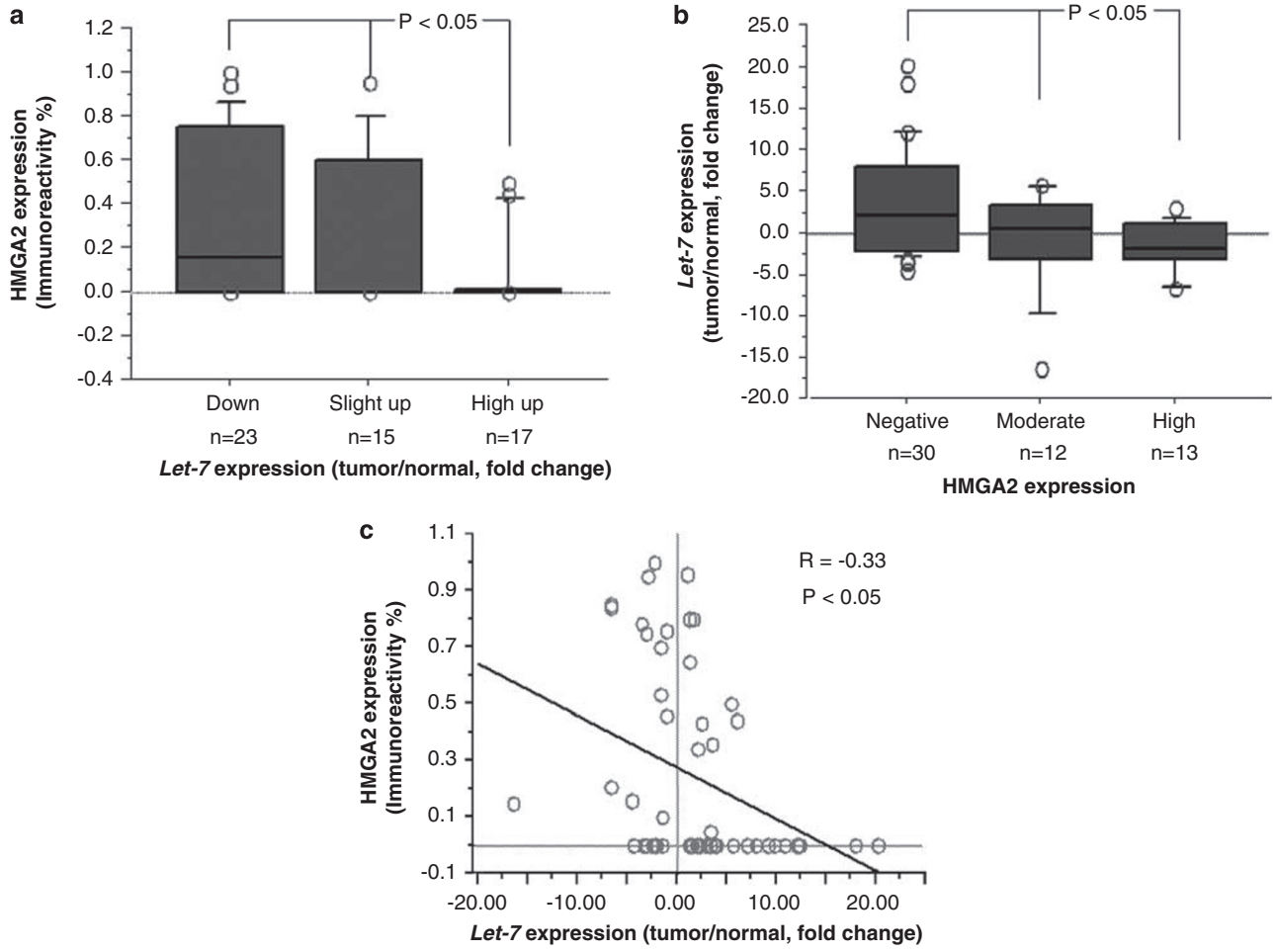

Figure 3 *Let-7* expression was negatively associated with HMGA2 immunoreactivity in pituitary adenomas. (**a**) HMGA2 expression levels were significantly higher in *let-7* downregulated adenomas than in *let-7* slightly upregulated adenomas and highly upregulated adenomas ($P<0.05$, Kruskal–Wallis test). (**b**) The significantly lower expression of *let-7* was detected in pituitary adenomas with high HMGA2 expression ($P<0.05$, Kruskal–Wallis test). (**c**) A significant inverse correlation between the expression of *let-7* and HMGA2 in pituitary adenomas was demonstrated using Pearson's correlation coefficient analysis ($r=-0.33$, $P<0.05$).

contributed to HMGA2 overexpression in those cases; other miRNAs such as *mir-98* may also relate to HMGA2 regulation in pituitary adenomas.[21] We noted that HMGA2 expression has been rarely detected in adenomas with high levels of *let-7*. Similar finding also has been discussed in small leiomyomas.[44] But HMGA2 expression has been detected in about half of adenomas with normal or a little high expression of *let-7*. These findings implied that more *let-7* may be needed in tumor than in normal tissue for effectively regulating target genes.

In conclusion, we found no immunoreactivity for HMGA2 in normal adult anterior pituitary glands. Nuclear expression of HMGA2 is common in the major types of pituitary adenomas with the exception of GH or mixed GH-PRL cell adenomas. High levels of expression of HMGA2 correlated with tumor grade, extent of invasion, tumor size, and higher Ki-67 proliferation index. These data support the premise that HMGA2 overexpression is an important and frequent event in pituitary tumorigenesis and be involved in tumor cell proliferation and tumor progression. In addition, we provided here pathological evidence of a significant link between HMGA2 oncogene and *let-7* miRNA. Loss and reduction of *let-7* expression may contribute to elevated HMGA2 protein expression, resulting in pituitary tumorigenesis and progression. Future studies should be directed at detecting exact role of *let-7* in each type of pituitary adenomas and uncovering the mechanism of *let-7* aberrant expression in pituitary adenomas. Furthermore, *let-7* miRNAs may be used as a novel anticancer agent in HMGA2-based therapy. To translate this finding into therapeutic strategy is a big challenge.

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