CLINICAL STUDY

Upregulation of *CDKN2A* and suppression of cyclin D1 gene expressions in ACTH-secreting pituitary adenomas

Yuji Tani, Naoko Inoshita¹, Toru Sugiyama, Masako Kato, Shozo Yamada², Masayoshi Shichiri³ and Yukio Hirata

Department of Clinical and Molecular Endocrinology, Tokyo Medical and Dental University Graduate School, 1-5-45, Yushima, Bunkyo-ku, Tokyo

113-8519, Japan, Departments of ¹Pathology and ²Hypothalamic and Pituitary Surgery, Toranomon Hospital, Tokyo 105-8470, Japan and ³Department of Endocrinology, Diabetes and Metabolism, School of Medicine, Kitasato University, Kanagawa 252-0375, Japan

(Correspondence should be addressed to Y Hirata; Email: yhirata.cme@tmd.ac.jp)

Abstract

Objective: Cushing's disease (CD) is usually caused by ACTH-secreting pituitary microadenomas, while silent corticotroph adenomas (SCA) are macroadenomas without Cushingoid features. However, the molecular mechanism(s) underlying their different tumor growth remains unknown. The aim of the current study was to evaluate and compare the gene expression profile of cell cycle regulators and cell growth-related transcription factors in CD, SCA, and non-functioning adenomas (NFA).

Design and methods: Tumor tissue specimens resected from 43 pituitary tumors were studied: CD ($n=10$), SCA ($n=11$), and NFA ($n=22$). The absolute transcript numbers of the following genes were quantified with real-time quantitative PCR assays: *CDKN2A* (or $p16^{INK4a}$), cyclin family (*A1*, *B1*, *D1*, and *E1*), *E2F1*, *RB1*, *BUB1*, *BUBR1*, *ETS1*, and *ETS2*. Protein expressions of p16 and cyclin D1 were semi-quantitatively evaluated by immunohistochemical study.

Results and conclusion: *CDKN2A* gene expression was about fourfold greater in CD than in SCA and NFA. The gene expressions of cyclins *D1*, *E1*, and *B1*, but not of *A1*, in CD were significantly suppressed than those in NFA. Cyclin *D1* gene expression positively correlated with cyclins *B1* and *E1*. The gene expressions of *E2F1*, *RB1*, *BUB1*, *BUBR1*, *ETS1*, and *ETS2* did not differ between each group. Positive immunostaining for p16 and negative immunostaining for cyclin D1 were more frequent in CD than in NFA; there were positive correlations between mRNA and protein expressions of p16 and cyclin D1. Thus, it is suggested that upregulated *CDKN2A* with the concomitant downregulated cyclin gene family is partly involved in the small size of ACTH-secreting adenoma.

European Journal of Endocrinology 163 523–529

Introduction

ACTH-secreting pituitary tumors causing Cushing's disease (CD) with a resultant hypercortisolism are usually microadenomas (1). In contrast, silent corticotroph adenomas (SCA) associated with positive immunoreactivity for ACTH, but not associated with signs and symptoms of Cushing's syndrome, present as macroadenomas (2) partly due to the delayed diagnosis resulting from the hormonal inactivity. However, the differences in molecular mechanisms underlying cell cycle regulation between ACTH-secreting microadenoma and SCA remain incompletely characterized.

Cyclin D1-induced activation of cyclin-dependent kinases (CDK4 and CDK6) is one of the most important mechanisms in the G₁ to S phase transition of the cell cycle, which causes phosphorylation of retinoblastoma protein (pRb) and subsequent release of E2F transcription factors to induce the expression of genes required for the S phase entry (3). Cyclin-CDK complexes are regulated by a family of kinase inhibitors that prevent phosphorylation of the corresponding substrates (4). Cyclin-dependent

kinase inhibitor 2A (*CDKN2A* or $p16^{INK4a}$) is a member of the protein family that specifically inhibits cyclin D-dependent kinases, which leads to the suppression of phosphorylation and activation of pRb (5). It has been reported that *CDKN2A* is mutated or inactivated in a variety of primary tumors and cancer cell lines (6, 7). Moreover, overexpression of *CDKN2A* has been shown to cause G₁ arrest in pituitary tumor cells (8), and an inverse association between *CDKN2A* and pRb alterations was observed in certain human cancers (9, 10). Recent molecular analysis revealed deregulation of cell cycle in human pituitary tumorigenesis (11); aberrations of one or more components of the pRb/*CDKN2A*/cyclin D1/CDK4 pathway seem to be a frequent event in pituitary adenoma (12–14). The most common mechanism of pRb/*CDKN2A* inactivation involves methylation in their gene promoter region.

However, the expression profile of cell cycle regulatory genes, such as retinoblastoma 1 (*RB1*) and *CDKN2A*, in pituitary corticotroph adenoma is still controversial regardless of methylation status, and the deranged expression of *CDKN2A* gene in relation to the

clinical characteristics of human pituitary tumors has not been investigated thus far.

Therefore, we studied the differential expression profile of cell cycle regulators and cell growth-related transcription factors in CD and SCA, and compared with those in non-functioning adenomas (NFA) using a real-time RT-PCR and immunohistochemical study.

Patients and methods

Patients

Forty-three patients (15 males and 28 females, mean age 51.2 ± 2.1 years), who underwent transsphenoidal surgery for removal of pituitary tumors at the Department of Hypothalamic and Pituitary Surgery, Toranomon Hospital and Tokyo Medical and Dental University, were studied. Informed consent was obtained from each patient before surgery. Tumor size was evaluated by the largest tumor diameter on magnetic resonance imaging. Blood samples were obtained in a resting supine position at 0800 h after overnight fast on three separate occasions. Plasma ACTH and serum cortisol levels were measured by IRMA and enzyme immunoassay respectively; the average values were used. Pituitary corticotroph adenoma was defined as ACTH-secreting pituitary adenoma causing CD, SCA as ACTH-positive pituitary adenomas without clinical stigmata of Cushing's syndrome as originally described (15), and NFA as clinically non-functioning pituitary adenomas other than SCA.

Real-time quantitative PCR assay

The tissue specimens were prepared as described previously (16). Total RNA was extracted from pituitary tumors (30–200 mg), and 5 µg total RNA were reverse transcribed. The transcripts of the following genes were quantified with Chromo4-based (Bio-Rad Laboratories) real-time RT-PCR using fluorescent SYBR Green Technology essentially as described (16, 17). A sequence-specific standard curve generated using 10-fold serial dilutions (10^7 – 10^3 copies per capillary reaction) of the standard PCR product of each target gene, which had been purified by Wizard SV Gel and PCR Clean-Up System (Promega), was included in each run to determine the absolute gene copy number per microgram RNA. Universal Probe Library System (Roche) combined with TaqMan Technology which prevents generation of non-specific amplification products was used to quantify the following genes: *CDKN2A*, E2F transcription factor 1 (*E2F1*), *RB1*, cyclin family (cyclins *A1*, *B1*, *D1*, and *E1*), budding uninhibited by benzimidazoles 1 homolog family (*BUB1* and *BUBR1*), E-twenty-six family (*ETS1* and *ETS2*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and β -actin. The PCR primers were synthesized by Greiner Bio-One (Tokyo, Japan), and their sequences are shown in Table 1. Non-specific amplification in the SYBR Green assay was estimated from typical amplification curve, such as very high quantification results, early C_t value, and/or the linearity of the serially diluted purified cDNA standard. The cDNAs of all tumor

Table 1 PCR primers used for real-time RT-PCR.

Primers	Probe	Sequences	PCR product size (bp)
<i>CDKN2A</i> (<i>p16^{INK4a}</i>)	#34	Forward: gtggacctggctgaggag Reverse: cttcaatcggggatgtctg	132
<i>E2F1</i>	#5	Forward: tccaagaaccacatccagtg Reverse: ctgggtcaaccctcaag	75
<i>RB1</i>	#55	Forward: tcaccttgaaatcgctgtcc Reverse: tggagatcttacaggagaaagataca	88
<i>CCNA1</i> (Cyclin <i>A1</i>)	#71	Forward: tcagtaccttagggaagctgaaa Reverse: ccagtccaccagaatcgtg	103
<i>CCNB1</i> (Cyclin <i>B1</i>)	#18	Forward: catggtgcactttcctcctt Reverse: aggtaatgttagagttggtgtcc	102
<i>CCND1</i> (Cyclin <i>D1</i>)	#67	Forward: gaagatcgctgccacctg Reverse: gacctcctcctcgacttct	61
<i>CCNE1</i> (Cyclin <i>E1</i>)	#36	Forward: ggccaaaatcgacaggac Reverse: gggctctgcacagactgcat	75
<i>BUB1</i>	#69	Forward: ggagaacgctctgtcagca Reverse: tccaaaaactcttcagcatgag	71
<i>BUBR1</i>	#7	Forward: gaaggacaaaagatcctggcta Reverse: tgtgctaatactgtataccaaaca	74
<i>ETS1</i>	#50	Forward: ccatcatcaagacggaaaaag Reverse: gggacatctgcacattccata	70
<i>ETS2</i>	#20	Forward: cccctgtggctaacagttaca Reverse: ggacccatcaaagggtgtcaa	68
<i>GAPDH</i>	#60	Forward: gctctctgctcctcctgttc Reverse: acgaccaaatccgttgactc	115
β -Actin	#64	Forward: ccaaccgcgagaagatga Reverse: tccatcacgatgccagtg	121

Figure 1 Gene expression profiles of cyclin family in CD, SCA, and NFA. Transcript copy numbers per microgram RNA of (a) cyclin A1, (b) cyclin B1, (c) cyclin D1, and (d) cyclin E1, as determined by real-time quantitative RT-PCR are shown. Each point with bars shows mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

specimens were prepared in a single run. Failure in RNA quality and/or RT reaction was estimated from very low transcript results. Such cDNA samples were subjected to repetition of RNA re-extraction and quantification, and then assessed using TaqMan PCR protocol.

Immunohistochemistry

Immunostaining was performed by the avidin–biotin–peroxidase complex (ABC) method using an autostaining machine (Ventana Benchmark HX System, Ventana Medical System, Tucson, AZ, USA). Primary antibodies used in this study were mouse monoclonal anti-p16 (clone JC8, Santa Cruz, San Diego, CA, USA; diluted 1:200) and rabbit monoclonal anti-cyclin D1 (clone SP4, Dako, Glostrup, Denmark; diluted 1:100). Hematoxylin was used as counterstain. The resulting sections were examined blind, without any prior knowledge of pituitary subtype by a single observer. The percentage of nuclear staining of p16 and cyclin D1 was evaluated semi-quantitatively as follows: p16, (–) negative staining; (1+) <20%; (2+) 20–50%; (3+) >50% and cyclin D1, (–) <10%; (+) 10–50%; (2+) 50–70%, (3+) >70%.

Statistical analyses

Data are expressed as means $\pm$ S.E.M. Differences between groups were examined for statistical significance using Kruskal–Wallis test with Dunn's *post-hoc* test. The relations between two continuous variables

were evaluated using Spearman's rank correlation coefficient. P values < 0.05 were considered statistically significant. All the statistical analyses were performed using Windows software Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

Results

Clinical characteristics and laboratory data of the patients

Based on clinical, endocrinological, and immunohistochemical studies, 10 patients (one male and nine females, aged 43.8 ± 4.3 years) were diagnosed to have CD, 11 (one male and 10 females, aged 50.6 ± 3.8 years) SCA, and 22 (13 males and 9 females, aged 55.0 ± 2.8 years) NFA.

The female to male ratio in CD and SCA was greater ($P < 0.001$) than that in NFA. There were no statistical differences in age among the three groups ($P = 0.12$). The tumor sizes in CD (10.4 ± 2.6 mm) were significantly ($P < 0.001$) smaller than those in SCA (40.2 ± 9.0 mm) and NFA (29.8 ± 1.6 mm). Both plasma ACTH levels (202.2 ± 38.9 pg/ml) and serum cortisol levels (33.3 ± 5.8 μ g/dl) in CD were significantly ($P < 0.001$) higher than those in SCA (ACTH, 31.0 ± 3.4 pg/ml and cortisol, 10.7 ± 1.1 μ g/dl) and NFA (ACTH, 26.2 ± 2.3 pg/ml and cortisol, 12.6 ± 0.8 μ g/dl) respectively.

Expression profile of cyclin family

We first quantified mRNA copy numbers of cyclin family in CD, SCA, and NFA (Fig. 1). Copy number of cyclin A1 mRNAs did not differ among the three groups, while that of cyclin B1 mRNAs in NFA ($1.8 \pm 1.0 \times 10^6$) was greater than that in CD ($6.5 \pm 4.2 \times 10^4$) ($P < 0.001$) and SCA ($2.2 \pm 0.6 \times 10^5$) ($P < 0.05$). Copy numbers of cyclin D1 mRNAs in SCA ($8.1 \pm 2.0 \times 10^6$) and NFA ($10.6 \pm 2.6 \times 10^6$) were markedly ($P < 0.001$) greater than those in CD ($7.7 \pm 2.4 \times 10^5$), while that of

Figure 2 Correlations of cyclin gene expression in CD, SCA, and NFA. Univariate positive correlations of cyclin D1 mRNA levels with those of (a) cyclin B1 and (b) cyclin E1 are shown.

cyclin *E1* mRNAs in NEA ($11.9 \pm 1.9 \times 10^4$) were significantly greater than those in CD ($4.9 \pm 1.4 \times 10^4$) ($P < 0.05$). There were significant positive correlations between cyclin *B1* and cyclin *D1* ($r = 0.60$, $P < 0.0001$), as well as between cyclin *D1* and cyclin *E1* ($r = 0.58$, $P < 0.0001$) mRNA levels (Fig. 2). We also performed analyses between tumors of comparable size by excluding the largest SCA/NEAs and the smallest CDs. There were similar differences in cyclins/CDKIs expressions among the three groups after matching for the tumor size.

Expression profile of genes related to RB pathway

We next studied the expression profile of genes interacting with RB pathway. Copy number of cell cycle inhibitor *CDKN2A* mRNAs in CD ($1.4 \pm 0.3 \times 10^6$) was significantly ($P < 0.05$) greater than that in SCA ($2.0 \pm 0.5 \times 10^5$) and NEA ($3.8 \pm 0.9 \times 10^5$), whereas copy number of the transcription factor *E2F1* or *RB1* did not show any significant differences among the three groups (Fig. 3).

The expressions of neither transcription family (*ETS1* and *ETS2*) regulating *CDKN2A* gene nor the cell cycle checkpoint genes (*BUB1* and *BUBR1*) showed any significant differences among the three tumor groups (data not shown). The same results were obtained after matching for the tumor size.

Figure 3 Gene expression profiles of cell cycle inhibitor and transcription factors in CD, SCA, and NEA. Transcript copy numbers per microgram RNA of (a) *CDKN2A*, (b) *E2F1*, and (c) *RB1*, as determined by real-time quantitative RT-PCR are shown as in Fig. 1. * $P < 0.05$.

Figure 4 Immunohistochemical analysis of *CDKN2A* and cyclin *D1* in pituitary tumors. Typical immunostainings for *CDKN2A* (left panels) and cyclin *D1* (right panels) are shown in CD (A and B), SCA (C and D), and NEA (E and F) (magnification $\times 100$). Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-10-0245>.

CDKN2A and cyclin *D1* immunohistochemistry and protein expression

As shown in Fig. 4 and Table 2, positive immunostaining cells for *CDKN2A* was observed in 8 out of 9 (89%) cases in CD, 5 out of 8 (63%) cases in SCA, and 4 out of 15 (27%) cases in NEA. In contrast, positive immunostaining cells for cyclin *D1* was observed in only 2 out of 9 (22%) cases in CD, but in all cases (100%) in SCA and NEA. Based on the immunohistochemical semi-quantitative grading score of *CDKN2A* and cyclin *D1* (Table 2), there were significant differences of copy numbers of mRNA expression between the groups (*CDKN2A*: $P < 0.04$; cyclin *D1*: $P < 0.01$). Copy number of *CDKN2A* mRNA ($1.4 \pm 0.4 \times 10^6$) in (2+) group was significantly ($P < 0.05$) greater than that ($1.7 \pm 0.4 \times 10^5$) in (−) group, while copy number of cyclin *D1* mRNA ($4.6 \pm 0.6 \times 10^6$) in (3+) group was significantly ($P < 0.01$) greater than that ($8.0 \pm 3.5 \times 10^5$) in (−) group (Fig. 5).

Discussion

By real-time quantitative RT-PCR, our study revealed the gene expression profile of a variety of cell cycle regulators and cell growth-related transcription factors

Table 2 CDKN2A and cyclin D1 expressions as evaluated by immunohistochemical study.

Tumor type (no. of cases)	Protein expression			
	(–)	1+	2+	3+
(A) CDKN2A				
CD (9)	1	3	4	1
SCA (8)	3	3	2	0
NFA (15)	11	4	0	0
(B) Cyclin D1				
CD (9)	7	2	0	0
SCA (8)	0	0	3	5
NFA (15)	0	0	3	12

The percentage of nuclear staining of CDKN2A was evaluated as follows: (–) negative staining, (1+) <20%, (2+) 20–50%, (3+) >50%. The percentage of nuclear staining of cyclin D1 was evaluated as follows: (–) <10%, (1+) 10–50%, (2+) 50–70%, (3+) >70%. CD, ACTH-secreting pituitary tumors causing Cushing's disease; SCA, silent corticotroph adenoma; NFA, non-functioning pituitary adenoma.

in ACTH-secreting tumors causing CD to compare with those in SCA and NFA. We have established highly sensitive quantification protocols on many different human genes in our laboratory (16, 17); our quantification experiments using both SYBR Green and TaqMan real-time RT-PCR protocols are more accurate than the conventional RT-PCR methods. The amplification curve of each SYBR Green-based PCR was carefully reviewed to negate the presence of non-specifically amplified products, and the absolute copy numbers per microgram RNA were calculated after confirming the close linearity of serially diluted purified target cDNA. Insufficient quality of RNA and failure of RT were easily detected by quantifying a wide spectrum of genes, including 'house-keeping' genes, not restricted to cell cycle regulators and transcription factors. Additionally, the conventional RT-PCR methods widely used to

evaluate the gene expression in most studies thus far reported simply 'normalize' the quantification results by house-keeping genes which, however, are influenced by several factors, thereby yielding erroneous results. Such technical maneuvers are absolutely important to draw any definite conclusion from the gene expression analysis of the resected tumors obtained during surgery, such as ACTH-secreting pituitary microadenomas.

Recent molecular analyses of human pituitary neoplasias showed deregulation of cell cycle in pituitary tumorigenesis, such as altered CDK regulation (11) and suppressed *CDKN2A* gene expression (18). It has been reported that cyclin D1 is often overexpressed in pituitary tumors (19), with its allelic imbalance in some tumor samples being analyzed (20). It has also been reported that a single null *CDKN2A* phenotype did not result in pituitary tumorigenesis (21), and that *CDKN2A* hypermethylation was the most common epigenetic deregulation in these neoplasias (12, 22). As for subtypes of pituitary adenoma, some reports have shown that clinically non-functioning macroadenoma has impaired *CDKN2A* expression compared with other pituitary tumor subtypes (23, 24), while other reports have shown that protein expression of p16 was down-regulated in all the pituitary tumors (18), and that high frequency of *CDKN2A* methylation did not differ between non-functioning and functioning adenomas (25). However, in the pituitary corticotroph adenomas in particular, the relationship between tumor size and the expression of *CDKN2A* has been undetermined yet.

Indeed, the hormonal inactivity allows the tumor to grow to a large size as a macroadenoma until the clinical manifestations develop in SCA and NFA, whereas hormonally active tumors are diagnosed earlier as a microadenoma due to the overt symptoms and signs of hypercortisolism. This may be another logical explanation of high incidence of microadenoma causing CD. However, the present study clearly showed enhanced *CDKN2A* gene expression concomitant with markedly suppressed cyclin *D1* gene expression in ACTH-secreting pituitary tumors causing CD. Such change could decrease the activation of CDK4, thereby leading to suppression of pRb phosphorylation. In other words, the reduced *CDKN2A* expression representing removal of 'cell cycle brake' in SCA and NFA may lead to unchecked cell proliferation. Moreover, the upregulation of *CDKN2A* expression in corticotroph adenomas could partly contribute to the reduced cyclin genes expression along with epigenetic inactivation of cyclin *D1* gene, thereby leading to the repression of cell cycles and tumor growth. This hypothesis is consistent with a previous study showing that *CDKN2A*-negative pituitary tumors were larger than *CDKN2A*-positive ones (24), and that oncogenic aberrations of *CDKN2A* and cyclin *D1* cooperate to deregulate *G₁* control (26). Thus, these results could at least in part account for the mechanism(s) for the 'small size' of ACTH-secreting adenoma compared to SCA and NFA.

Figure 5 Correlations of gene and protein expressions of p16 and cyclin D1 in CD, SCA, and NFA. Correlations between mRNA levels by real-time quantitative RT-PCR and protein expression by immunohistochemical semi-quantitative grading score are shown for (a) CDKN2A and (b) cyclin D1. There were significant differences between the groups: CDKN2A ($P < 0.04$) and cyclin D1 ($P < 0.01$). Dunn's *post hoc* test compared with each group: * $P < 0.05$ and ** $P < 0.01$.

Recent report has shown that cyclin E is significantly increased in corticotroph adenomas (19), speculating that overexpression of cyclin E is relevant to low expression of p27 in corticotroph adenomas. In contrast, our study clearly showed that cyclin E1 expression is much reduced in corticotroph adenomas and correlated well with cyclin D1 expression, suggesting that overexpression of cyclin D1 may lead to E2F-mediated cyclin E1 transcription (4), which could be regulated by p16 rather than by p27. Such contradictory results could be accounted for by the complex mechanism(s) of CKIs regulation involved and/or the different methods (RT-PCR versus immunohistochemical analysis) employed in each study.

A pivotal role of pRb inactivation in pituitary tumorigenesis has been well recognized, such as loss of heterozygosity (27) and decreased expression with hypermethylation of its promoter (28). Our study showed no apparent differences in RB1 at its gene expression level, although a possible involvement of pRb activation in the mechanism of growth inhibition in corticotroph adenomas could not be excluded. However, it is reasonable to assume that the increased expression of cyclin D1 leads to hyperphosphorylation of pRb via a cyclin D1/CDK4 complex.

Neither transcription factors (*ETS1* and *ETS2*) known to activate the *CDKN2A* promoter nor mitotic check point genes (*BUB1* and *BUBR1*) revealed any differences among the three tumor groups, suggesting a negative involvement of the altered expression of such genes in corticotroph adenomas.

The exact mechanism(s) of concomitantly down-regulated cyclin family (*D1*, *E1* and *B1*) and upregulated *CDKN2A* gene in ACTH-secreting pituitary tumors causing CD as demonstrated in our study remains unsettled. It has been reported that glucocorticoids suppress the growth of murine corticotrophic tumor cells (AtT20) *in vitro* (29), and that the size of the corticotroph adenomas correlates with the degree of insensitivity to glucocorticoid feedback in dogs *in vivo* (30). Consistent with these studies, our study showed that ACTH-secreting pituitary adenomas were smaller than SCA and NFA, suggesting that cortisol excess may have contributed to the size differences. Furthermore, promoter region of *CDKN2A* contains putative binding sites for CEBPB (C/EBP β), which is induced by glucocorticoid and mediates its effect (31). Collectively, glucocorticoid excess may directly contribute to the upregulation of *CDKN2A* in ACTH-secreting pituitary tumors, although the correlation between *CDKN2A* expression and circulating cortisol level was not significant in our study (unpublished observation). Thus, further study using larger sample size of CD patients is required. Moreover, our data with a significant reduced expression of cyclin D1 in ACTH-secreting pituitary tumors are consistent with those of other studies showing that glucocorticoids inhibit cell

proliferation by virtue of their ability to downregulate cyclin D expression in lymphoid cell (32).

In conclusion, our data revealed for the first time the enhanced expression of *CDKN2A* with concomitant suppressed expression of cyclin family (*D1*, *E1*, and *B1*) in ACTH-secreting pituitary tumors causing CD, which may in part contribute to the small tumor size. However, it remains to be determined whether glucocorticoid-induced gene expression of *CDKN2A* is related to epigenetic inactivation of cyclin *D1* gene, and whether inactivation of pRb pathway is related to reduced growth rate of corticotroph adenomas.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

Acknowledgements

This study was supported in part by the Grant-in-aid from the Ministry of Education, Science, Sports and Culture, and the Ministry of Health, Welfare and Labor of Japan. We thank Dr T Tateno for his cooperation in this study.

References

- 1 Boscaro M, Barzon L, Fallo F & Sonino N. Cushing's syndrome. *Lancet* 2001 **357** 783–791. (doi:10.1016/S0140-6736(00)04172-6)
- 2 Horvath E, Kovacs K, Killinger DW, Smyth HS, Platts ME & Singer W. Silent corticotrophic adenomas of the human pituitary gland: a histologic, immunocytologic, and ultrastructural study. *American Journal of Pathology* 1980 **98** 617–638.
- 3 Harbour JW & Dean DC. The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes and Development* 2000 **14** 2393–2409. (doi:10.1101/gad.813200)
- 4 Sherr CJ & Roberts JM. CDK inhibitors: positive and negative regulators of G₁-phase progression. *Genes and Development* 1999 **13** 1501–1512. (doi:10.1101/gad.13.12.1501)
- 5 Serrano M, Hannon GJ & Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993 **366** 704–707. (doi:10.1038/366704a0)
- 6 Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB & Sidransky D. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nature Medicine* 1995 **1** 686–692. (doi:10.1038/nm0795-686)
- 7 Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D & Baylin SB. Inactivation of the *CDKN2/p16/MTS1* gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Research* 1995 **55** 4525–4530.
- 8 Frost SJ, Simpson DJ, Clayton RN & Farrell WE. Transfection of an inducible p16/CDKN2A construct mediates reversible growth inhibition and G₁ arrest in the AtT20 pituitary tumor cell line. *Molecular Endocrinology* 1999 **13** 1801–1810. (doi:10.1210/me.13.11.1801)

- 9 Aagaard L, Lukas J, Bartkova J, Kjerulff AA, Strauss M & Bartek J. Aberrations of p16^{Ink4} and retinoblastoma tumour-suppressor genes occur in distinct sub-sets of human cancer cell lines. *International Journal of Cancer* 1995 **61** 115–120. (doi:10.1002/ijc.2910610120)
- 10 Szijan I, Orlov I, Dalamon V, Vergani P, Danilowicz K, Mezzadri N, Cordon-Cardo C & Bruno OD. Alterations in the retinoblastoma pathway of cell cycle control in parathyroid tumors. *Oncology Reports* 2000 **7** 421–425.
- 11 Malumbres M & Barbacid M. To cycle or not to cycle: a critical decision in cancer. *Nature Reviews. Cancer* 2001 **1** 222–231. (doi:10.1038/35106065)
- 12 Yoshino A, Katayama Y, Ogino A, Watanabe T, Yachi K, Ohta T, Komine C, Yokoyama T & Fukushima T. Promoter hypermethylation profile of cell cycle regulator genes in pituitary adenomas. *Journal of Neuro-Oncology* 2007 **83** 153–162. (doi:10.1007/s11060-006-9316-9)
- 13 Simpson DJ, Frost SJ, Bicknell JE, Broome JC, McNicol AM, Clayton RN & Farrell WE. Aberrant expression of G(1)/S regulators is a frequent event in sporadic pituitary adenomas. *Carcinogenesis* 2001 **22** 1149–1154. (doi:10.1093/carcin/22.8.1149)
- 14 Musat M, Morris DG, Korbonits M & Grossman AB. Cyclins and their related proteins in pituitary tumorigenesis. *Molecular and Cellular Endocrinology*. 2010. (doi:10.1016/j.mce.2010.03.017)
- 15 Kovacs K, Horvath E, Bayley TA, Hassaram ST & Ezrin C. Silent corticotroph cell adenoma with lysosomal accumulation and crinophagy. A distinct clinicopathologic entity. *American Journal of Medicine* 1978 **64** 492–499. (doi:10.1016/0002-9343(78)90236-X)
- 16 Tateno T, Izumiyama H, Doi M, Yoshimoto T, Shichiri M, Inoshita N, Oyama K, Yamada S & Hirata Y. Differential gene expression in ACTH-secreting and non-functioning pituitary tumors. *European Journal of Endocrinology* 2007 **157** 717–724. (doi:10.1530/EJE-07-0428)
- 17 Shichiri M, Yoshinaga K, Hisatomi H, Sugihara K & Hirata Y. Genetic and epigenetic inactivation of mitotic checkpoint genes hBUB1 and hBUBR1 and their relationship to survival. *Cancer Research* 2002 **62** 13–17.
- 18 Woloschak M, Yu A, Xiao J & Post KD. Frequent loss of the P16^{Ink4a} gene product in human pituitary tumors. *Cancer Research* 1996 **56** 2493–2496.
- 19 Jordan S, Lidhar K, Korbonits M, Lowe DG & Grossman AB. Cyclin D and cyclin E expression in normal and adenomatous pituitary. *European Journal of Endocrinology* 2000 **143** R1–R6. (doi:10.1530/eje.0.143R001)
- 20 Hibberts NA, Simpson DJ, Bicknell JE, Broome JC, Hoban PR, Clayton RN & Farrell WE. Analysis of cyclin D1 (CCND1) allelic imbalance and overexpression in sporadic human pituitary tumors. *Clinical Cancer Research* 1999 **5** 2133–2139.
- 21 Quereda V & Malumbres M. Cell cycle control of pituitary development and disease. *Journal of Molecular Endocrinology* 2009 **42** 75–86. (doi:10.1677/JME-08-0146)
- 22 Simpson DJ, Bicknell JE, McNicol AM, Clayton RN & Farrell WE. Hypermethylation of the p16/CDKN2A/MTSI gene and loss of protein expression is associated with nonfunctional pituitary adenomas but not somatotrophinomas. *Genes, Chromosomes and Cancer* 1999 **24** 328–336. (doi:10.1002/(SICI)1098-2264(199904)24:4<328::AID-GCC6>3.0.CO;2-P)
- 23 Machiavelli G, Cotignola J, Danilowicz K, Carbonara C, Paes de Lima A, Basso A, Bruno OD & Szijan I. Expression of p16(INK4A) gene in human pituitary tumours. *Pituitary* 2008 **11** 71–75. (doi:10.1007/s11102-007-0077-z)
- 24 Seemann N, Kuhn D, Wrocklage C, Keyvani K, Hackl W, Buchfelder M, Fahlbusch R & Paulus W. CDKN2A/p16 inactivation is related to pituitary adenoma type and size. *Journal of Pathology* 2001 **193** 491–497. (doi:10.1002/path.833)
- 25 Ogino A, Yoshino A, Katayama Y, Watanabe T, Ota T, Komine C, Yokoyama T & Fukushima T. The p15(INK4b)/p16(INK4a)/RB1 pathway is frequently deregulated in human pituitary adenomas. *Journal of Neuropathology and Experimental Neurology* 2005 **64** 398–403.
- 26 Lukas J, Aagaard L, Strauss M & Bartek J. Oncogenic aberrations of p16INK4/CDKN2 and cyclin D1 cooperate to deregulate G₁ control. *Cancer Research* 1995 **55** 4818–4823.
- 27 Pei L, Melmed S, Scheithauer B, Kovacs K, Benedict WF & Prager D. Frequent loss of heterozygosity at the retinoblastoma susceptibility gene (RB) locus in aggressive pituitary tumors: evidence for a chromosome 13 tumor suppressor gene other than RB. *Cancer Research* 1995 **55** 1613–1616.
- 28 Simpson DJ, Hibberts NA, McNicol AM, Clayton RN & Farrell WE. Loss of pRb expression in pituitary adenomas is associated with methylation of the RB1 CpG island. *Cancer Research* 2000 **60** 1211–1216.
- 29 van Wijk PA, van Neck JW, Rijnberk A, Croughs RJ & Mol JA. Proliferation of the murine corticotrophic tumour cell line AtT20 is affected by hypophysiotrophic hormones, growth factors and glucocorticoids. *Molecular and Cellular Endocrinology* 1995 **111** 13–19. (doi:10.1016/0303-7207(95)03541-E)
- 30 Kooistra HS, Voorhout G, Mol JA & Rijnberk A. Correlation between impairment of glucocorticoid feedback and the size of the pituitary gland in dogs with pituitary-dependent hyperadrenocorticism. *Journal of Endocrinology* 1997 **152** 387–394. (doi:10.1677/joe.0.1520387)
- 31 Gotoh T, Chowdhury S, Takiguchi M & Mori M. The glucocorticoid-responsive gene cascade. Activation of the rat arginase gene through induction of C/EBPbeta. *Journal of Biological Chemistry* 1997 **272** 3694–3698. (doi:10.1074/jbc.272.30.18602)
- 32 Reisman D & Thompson EA. Glucocorticoid regulation of cyclin D3 gene transcription and mRNA stability in lymphoid cells. *Molecular Endocrinology* 1995 **9** 1500–1509. (doi:10.1210/me.9.11.1500)

Received 6 June 2010

Accepted 5 July 2010