CLINICAL STUDY

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

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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 p16INK4a), 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.

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 p16INK4a) 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

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

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<tr>
<th>Primers</th>
<th>Probe</th>
<th>Sequences</th>
<th>PCR product size (bp)</th>
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<tbody>
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<tr>
<td></td>
<td></td>
<td>Reverse: cttcaatctgggagtgctg</td>
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<tr>
<td>E2F1</td>
<td>#5</td>
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<td></td>
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<tr>
<td>β-Actin</td>
<td>#64</td>
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<td>Reverse: tccacaagatgtcagcttg</td>
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</table>
Gene expression profile in pituitary adenomas

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 μg/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 μg/dl) and NFA (ACTH, 26.2 ± 2.3 pg/ml and cortisol, 12.6 ± 0.8 μg/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 ± 1.0 × 10^6) was greater than that in CD (6.5 ± 4.2 × 10^5) (P < 0.001) and SCA (2.2 ± 0.6 × 10^5) (P < 0.05). Copy numbers of cyclin D1 mRNAs in SCA (8.1 ± 2.0 × 10^6) and NFA (10.6 ± 2.6 × 10^6) was markedly (P < 0.001) greater than those in CD (7.7 ± 2.4 × 10^5), while that of

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).

Statistical analyses

Data are expressed as means ± 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

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%.

Immunohistochemistry

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 ± S.E.M. *P < 0.05, **P < 0.01, and ***P < 0.001.

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 NFA (11.9 ± 1.9 × 10^4) were significantly greater than those in CD (4.9 ± 1.4 × 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/NFAs 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 \( (1.4 ± 0.3 \times 10^6) \) was significantly \( (P<0.05) \) greater than that in SCA \( (2.0 ± 0.5 \times 10^5) \) and NFA \( (3.8 ± 0.9 \times 10^5) \), 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.

**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 NFA. 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 NFA. 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 \( (1.4 ± 0.4 \times 10^6) \) in \( (+) \) group was significantly \( (P<0.05) \) greater than that \( (1.7 ± 0.4 \times 10^5) \) in \( (–) \) group, while copy number of cyclin D1 mRNA \( (4.6 ± 0.6 \times 10^6) \) in \( (+) \) group was significantly \( (P<0.01) \) greater than that \( (8.0 ± 3.5 \times 10^5) \) 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.

<table>
<thead>
<tr>
<th>Tumor type (no. of cases)</th>
<th>Protein expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(−)</td>
</tr>
<tr>
<td>(A) CDKN2A</td>
<td>CD (9)</td>
</tr>
<tr>
<td></td>
<td>SCA (8)</td>
</tr>
<tr>
<td></td>
<td>NFA (15)</td>
</tr>
<tr>
<td>(B) Cyclin D1</td>
<td>CD (9)</td>
</tr>
<tr>
<td></td>
<td>SCA (8)</td>
</tr>
<tr>
<td></td>
<td>NFA (15)</td>
</tr>
</tbody>
</table>

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 checkpoint 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 downregulated 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/EBPβ), 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.

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