Reduced expression levels of the cell-cycle inhibitor p27^{Kip1} in human pituitary adenomas

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Abstract

The molecular mechanisms leading to increased cellular proliferation rates and, thus, tumor formation in the anterior pituitary gland are poorly understood. The cyclin-dependent kinase inhibitor p27^{Kip1} is a key molecule regulating the G1 phase of the cell cycle in many cell types. Furthermore, it was shown that p27 knock-out mice develop pro-opiomelanocortin-positive pituitary tumors. In an effort to clarify the role of p27 in the normal and tumorous human pituitary, we studied the expression of p27 by immunohistochemistry, using a highly specific mouse monoclonal anti-human p27 antibody. Normal pituitaries and 54 pituitary adenomas (twelve somatotrope adenomas, nine prolactinomas, twelve corticotrope adenomas, three TSH-producing tumors, six gonadotrope adenomas, six null cell adenomas, and six oncocytomas) were analyzed. p27 expression was determined semiquantitatively with regard to both the percentage of positive cells and the intensity of the staining. Normal human pituitaries showed strong expression of p27 in most nuclei. In contrast, the levels of p27 were reduced in the majority of the tumors analyzed. Twenty-two tumors (six somatotrope adenomas, five prolactinomas, four corticotrope adenomas, two TSH-producing tumors, two gonadotrope adenomas, and three null cell adenomas) were completely p27-negative. In 18 tumors, p27 expression was found in $\leq 10\%$ of the cells. In the other ten tumors, 11–80% of the cells were p27-positive. In summary, we were able to demonstrate reduced expression levels of the cell-cycle inhibitor p27 in tumors derived from all pituitary cell types. Our data indicate that p27 may be an important regulator of cellular proliferation in the anterior pituitary, the underexpression of which could play a role in pituitary tumorigenesis.

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Introduction

The molecular processes underlying proliferation and differentiation of normal and tumorous pituitary cells are still incompletely understood (1). It has been demonstrated that pituitary adenomas are monoclonal in origin (2–6). In some tumors, somatic mutations have been shown to promote permanent activation of signal cascades involved in cellular proliferation. For instance, up to 40% of somatotrope tumors bear an activating mutation in the α -subunit of the Gprotein heterotrimer (7–10). Such mutations are rarely detected in other pituitary tumors (11, 12). Furthermore, the intracellular events downstream of membrane receptor-induced signal transduction cascades have not been analyzed in greater detail. This is especially true for the complex intranuclear machinery governing the cell cycle in normal and tumorous pituitary cells.

Progression through the eukaryotic cell cycle is regulated by cyclin-dependent kinases (Cdk) and their associated cyclins (13). G1 progression and G1/S transition depend on the sequential activation of cyclinD–Cdk4 or –Cdk6, cyclinE–Cdk2, and cyclinA–Cdk2 complexes (13). Cdk activity is tightly regulated by different mechanisms, including changes in cyclin and Cdk levels, phosphorylation of Cdks, and interaction of Cdks with a number of inhibitory factors, termed Cdk inhibitors (13). Because of their capacity to delay or even arrest progression through the cell cycle, Cdk inhibitors are recognized as important tumor suppressors (13, 14). Accordingly, the activity of these factors has been shown to be critically diminished in a large number of malignancies (15–17). Cdk inhibitors either belong to the class of Ink4 proteins (p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}), which contain ankyrin repeats, or to a structurally distinct class of proteins, which include p21^{Cip1/Waf1}, p27^{Kip1}, and p57^{Kip2} (13, 18, 19).

The latter primarily interact with cyclin–Cdk complexes controlling the G1 and S phase of the cell cycle (20).

p27 is widely expressed both in proliferating and differentiating cells, and can inhibit cyclinD–Cdk4, cyclinE–Cdk2, and cyclinA–Cdk2 complexes *in vitro* (15). *In vivo*, p27 predominantly counteracts the effects of cyclinE–Cdk2 complexes in response to various extra- and intracellular factors (21–23). Mice in which the p27 gene has been inactivated by homologous recombination display increased body size, multiorgan hyperplasia, and retinal dysplasia (24–26). Furthermore, these mice develop pro-opiomelanocortin (POMC)-positive tumors in the intermediate lobe of the pituitary (24–26). In order to investigate its potential role in human pituitary tumorigenesis, we therefore studied the expression of p27 in surgically removed hypophyseal tissue samples from patients with non-functioning and hormone-secreting pituitary adenomas.

Materials and methods

Tissue collection

Pathological and surgical specimens, which had been routinely fixed in 4% buffered formalin and embedded in paraffin, were used for immunocytochemistry. Tissue samples included five normal postmortem pituitaries and surgically removed pituitary adenomas (twelve somatotrope adenomas, nine prolactinomas, twelve corticotrope adenomas, three thyrotropin (TSH)-producing tumors, six gonadotrope adenomas, six null cell adenomas, and six oncocytomas) (Table 1). The material was selected following histological review from the files of the Department of Pathology, Marienkrankenhaus, Hamburg, Germany. The surgical specimens in these files were obtained from the Department of Neurosurgery, University Clinic Eppendorf, Hamburg, Germany. All lesions were classified according to the most recent WHO criteria.

Immunohistochemistry

Serial sections of 4–6 μm were cut from the paraffin blocks and mounted on amino propylethoxy-silane-coated slides, deparaffinized in xylene and rehydrated in graded alcohol to Tris-buffered saline (TBS; 50 mmol/l Tris, 150 mmol/l NaCl, pH 7.4). The slides were microwaved for 5 $\times$ 2 min in 10 mmol/l citrate, pH 6.0. After cooling down for 20 min, the slides were washed in TBS, blocked for 30 min at room temperature with normal goat serum (DAKO, Glostrup, Denmark), diluted 1:20 in TBS and incubated with a p27 mouse monoclonal antibody (Medac, Hamburg, Germany) at a dilution of 1:10 in TBS for 24 h. Non-immune murine serum (DAKO) at the same dilution was used for negative control. Slides were then reacted with biotin-labeled anti-mouse IgG and incubated with preformed avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA).

Diaminobenzidine substrate was then added in the presence of horseradish peroxidase. Sections were counterstained with hematoxylin, dehydrated and mounted.

Microscopic evaluation

Histological and immunohistochemical evaluation was performed independently by two pathologists. All tumors were routinely stained for anterior pituitary hormones. Nuclear staining for p27 was assessed on an arbitrary scale as absent (–), sparse (+), moderate (++) or strong (+++). Each tumor was analyzed with respect to the percentage of cells in the different categories.

Results

Immunohistochemical analysis of p27 expression in normal postmortem pituitaries as well as in the normal pituitary tissue adherent to some of the adenomas revealed moderate/strong nuclear staining in more than 80% of the cells ($5.0 \pm 5.5\%$ no staining, $10.0 \pm 9.4\%$ sparse staining, $41 \pm 5.5\%$ moderate staining, and $44 \pm 15.2\%$ strong staining) (mean $\pm$ s.d.). In contrast, p27 levels were found to be reduced in the majority of the tumors analyzed (Fig. 1 and Table 1). p27 underexpression was not restricted to a certain type of tumor; however, the mean percentage of p27-negative cells was highest in corticotrope adenomas and lowest in gonadotrope adenomas. In detail, the following results were obtained (Table 1).

In somatotrope adenomas, $87.6 \pm 19.0\%$ of the cells were p27-negative, $7.0 \pm 9.6\%$ showed sparse, $3.8 \pm 7.7\%$ moderate, and $1.7 \pm 3.9\%$ strong staining. In prolactinomas, $89.8 \pm 22.8\%$ of the cells showed no staining for p27, $4.1 \pm 6.9\%$ showed sparse, $3.9 \pm 9.9\%$ moderate, and $2.2 \pm 6.7\%$ strong staining. In corticotrope adenomas, $95.8 \pm 4.5\%$ of the cells did not express p27 at all, $2.9 \pm 3.1\%$ showed sparse, $1.3 \pm 2.3\%$ moderate, and 0% strong staining. In TSH-producing adenomas, $86.7 \pm 23.0\%$ of the cells were p27-negative, $10.0 \pm 17.3\%$ showed sparse, $3.3 \pm 5.8\%$ moderate, and 0% strong staining. Gonadotrope adenomas showed a tendency towards higher p27 expression levels as compared with the other tumor types. In gonadotrope adenomas, only $63.3 \pm 32\%$ of the cells were p27-negative, $16.7 \pm 13.7\%$ showed sparse, $16.7 \pm 16.3\%$ moderate, and $3.3 \pm 8.2\%$ strong staining. In null cell adenomas, $88.3 \pm 21.6\%$ of the cells did not show any p27 staining, $5.0 \pm 7.7\%$ showed sparse, $5.8 \pm 12.0\%$ moderate, and $0.8 \pm 2.0\%$ strong staining. Finally, in oncocytomas, $76.8 \pm 23.5\%$ of tumor cells were p27-negative, $13.1 \pm 11.8\%$ showed sparse, $8.3 \pm 9.8\%$ moderate, and $1.7 \pm 2.6\%$ strong staining for p27.

Discussion

In this study, we demonstrate strongly reduced expression levels of the Cdk inhibitor p27 in all types of human

Table 1 Expression levels of p27 in different types of anterior pituitary adenomas. Nuclear staining for p27 was assessed on an arbitrary scale as absent (–), sparse (+), moderate (++), or strong (+++). Each tumor was analyzed with respect to the percentage of cells in each category.

No.	Adenoma type	Percentage of cells			
		(–)	(+)	(++)	(+++)
1	Somatotrope, densely granulated	100	–	–	–
2	Somatotrope, densely granulated	96	2	2	–
3	Somatotrope, densely granulated	80	20	–	–
4	Somatotrope, densely granulated	80	20	–	–
5	Somatotrope, densely granulated	50	20	20	10
6	Somatotrope, densely granulated	50	20	20	10
7	Somatotrope, sparsely granulated	100	–	–	–
8	Somatotrope, sparsely granulated	100	–	–	–
9	Somatotrope, sparsely granulated	100	–	–	–
10	Somatotrope, sparsely granulated	100	–	–	–
11	Somatotrope, sparsely granulated	100	–	–	–
12	Somatotrope, sparsely granulated	95	2	3	–
13	Prolactinoma, densely granulated	100	–	–	–
14	Prolactinoma, densely granulated	100	–	–	–
15	Prolactinoma, densely granulated	90	5	5	–
16	Prolactinoma, sparsely granulated	100	–	–	–
17	Prolactinoma, sparsely granulated	100	–	–	–
18	Prolactinoma, sparsely granulated	100	–	–	–
19	Prolactinoma, sparsely granulated	98	2	–	–
20	Prolactinoma, sparsely granulated	90	10	–	–
21	Prolactinoma, sparsely granulated	30	20	30	20
22	Corticotrope, densely granulated	100	–	–	–
23	Corticotrope, densely granulated	100	–	–	–
24	Corticotrope, sparsely granulated	100	–	–	–
25	Corticotrope, sparsely granulated	100	–	–	–
26	Corticotrope, sparsely granulated	99	1	–	–
27	Corticotrope, densely granulated	98	2	–	–
28	Corticotrope, densely granulated	98	2	–	–
29	Corticotrope, densely granulated	95	5	–	–
30	Corticotrope, sparsely granulated	90	10	–	–
31	Corticotrope, densely granulated	90	5	5	–
32	Corticotrope, sparsely granulated	90	5	5	–
33	Corticotrope, sparsely granulated	90	5	5	–
34	TSH-producing adenoma	100	–	–	–
35	TSH-producing adenoma	100	–	–	–
36	TSH-producing adenoma	60	30	10	–
37	Gonadotrope adenoma	100	–	–	–
38	Gonadotrope adenoma	100	–	–	–
39	Gonadotrope adenoma	60	20	20	–
40	Gonadotrope adenoma	60	30	10	–
41	Gonadotrope adenoma	40	30	30	–
42	Gonadotrope adenoma	20	20	40	20
43	Null cell adenoma	100	–	–	–
44	Null cell adenoma	100	–	–	–
45	Null cell adenoma	100	–	–	–
46	Null cell adenoma	95	5	–	–
47	Null cell adenoma	90	5	5	–
48	Null cell adenoma	45	20	30	5
49	Oncocytoma	98	2	–	–
50	Oncocytoma	98	2	–	–
51	Oncocytoma	95	5	–	–
52	Oncocytoma	70	20	10	–
53	Oncocytoma	55	20	20	5
54	Oncocytoma	45	30	20	5

pituitary adenomas as compared with the normal human pituitary. In the majority of the tumors, p27 expression was found in less than 10% of the cells. In the remaining tumors, p27 expression was clearly below the levels found in normal pituitaries.

p27 has previously been shown to be underexpressed in a large number of human malignancies, including breast cancer (16) and colon cancer (17). More recently, we demonstrated pronounced underexpression of p27 in endometrial cancer (27). However, malignant tumors

Figure 1 Immunohistochemical detection of p27 in the normal human pituitary and in pituitary adenomas. (A) Most nuclei in the normal pituitary show moderate/strong staining for p27 (22 $\times$). (B) Absent expression of p27 in a somatotrope adenoma (case 5, 22 $\times$). (C) Representative portion of a partially p27-negative somatotrope adenoma (case 11, 22 $\times$). (D) Example of a p27-negative corticotrope adenoma (case 22, 22 $\times$).

rarely occur in the pituitary gland. Consequently, mutations and/or disregulated expression of oncogenes and/or tumor suppressor genes associated with malignant states, e.g. of ras, p53, or the retinoblastoma gene, are not frequently observed in pituitary adenomas (28–30). In this study, we have shown that p27 is underexpressed in benign pituitary tumors. As opposed to other tumor suppressors, downregulation of p27 thus seems to be important for cellular proliferation in both malignant and benign neoplasias. It will be crucial to determine whether low p27 levels are the primary cause for pituitary tumor formation, or whether they merely reflect increased proliferative activity induced by other factors. The former mechanism is suggested by the previously reported p27 knock-out experiments (24–26). These animals developed POMC-positive tumors of the intermediate lobe, indicating that p27 is involved in regulating proliferation in this cell type. However, the intermediate lobe is hypoplastic or absent in humans. Furthermore, in our study, underexpression of p27 was not restricted to POMC-expressing pituitary tumors in humans. Jin *et al.* (31) reported lower p27 levels in adrenocorticotropin-producing adenomas as compared

with other pituitary tumors. Nevertheless, p27 levels were still lower in the latter than in normal pituitaries. These data may, therefore, indicate species-specific differences in the intrapituitary role of p27.

What could be the mechanisms leading to downregulation of p27 in pituitary cells? Several investigators have analyzed whether mutations in the p27 gene could account for pituitary tumor development, yet no such mutations were detected in pituitary adenomas (32–34). This is consistent with previous findings in other types of neoplasias, including malignant tumors, in which p27 mutations were highly infrequent (35, 36). Downregulation of p27 in pituitary adenomas does not occur at the transcriptional level either, since p27 mRNA expression is not altered in these tumors (31, 34). Thus, translational and/or post-translational mechanisms are likely to be involved in downregulating p27 in the pituitary. In other cell types, it has been shown that the ubiquitin proteasome pathway is the principal system regulating the abundance of p27 (37). Future studies will have to deal with the question as to whether this pathway is also crucial for pituitary p27 expression, and how known pituitary growth and

growth-inhibiting factors, such as transforming growth factor- α (TGF α) (38, 39) and TGF β (22, 31), are involved in its regulation.

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