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Low Expression of the Cell Cycle Inhibitor p27^{Kip1} in Normal Corticotroph Cells, Corticotroph Tumors, and Malignant Pituitary Tumors

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ABSTRACT

The cell cycle is regulated by a number of inhibitors, including p27^{Kip1} (p27), which belongs to the kip1 family. By binding to the cyclin/cyclin-dependent kinase complexes, it regulates progression of G_1 to S phase in the cell cycle. It has been reported that p27 knockout mice develop multiorgan hyperplasia and intermediate lobe pituitary tumors secreting ACTH. Previously, we and others have been unable to show any consistent change in messenger RNA expression or genomic mutations for p27 in human corticotroph adenomas. However, dysregulation at the protein level has been reported in nonendocrine tumors, and we, therefore, investigated the expression of p27 in a range of benign and metastatic pituitary tumors. We studied a total of 107 pituitaries, including normal pituitary (n = 20), Cushing's disease (n = 21), acromegaly (n = 19), nonfunctioning adenomas (n = 18), prolactinomas (n = 7), TSH-omas (n = 2), FSH-omas (n = 6), aggressive tumors showing invasiveness and recurrence (n = 9), and metastatic pituitary carcinomas (n = 5). Using standard immunohistochemical techniques with a highly specific monoclonal antibody, p27 expression was determined quantitatively as the percentage of cells showing strongly positive, weak, or negative staining. In each sample, ~500 cells were analyzed. We also analyzed normal pituitaries using double-labeling for p27 and each of the pituitary hormones to characterize the expression of p27 in each cell type. p27 was expressed in normal pituitary cells; in tumors expressing GH, prolactin, TSH, and FSH; and in aggressive tumors, but markedly reduced expression of p27 was seen in corticotroph tumors and pituitary carcinomas. In the normal pituitary, somatotroph, lactotroph, and thyrotroph cells showed strong p27 staining, whereas normal corticotroph cells showed a much lower level of p27 staining ($P < 0.001$). Somatotroph, lactotroph, gonadotroph, and thyrotroph adenomas showed a lower level of p27 expression compared with normal somatotrophs ($P = 0.02$), lactotrophs ($P = 0.03$), gonadotrophs ($P = 0.01$), and thyrotrophs, respectively, whereas the lower level of p27 expression present in normal corticotrophs virtually disappeared in corticotroph adenomas ($P = 0.001$).

We conclude that pituitary adenomas show a lower level of p27 protein expression than the normal cells from which they are derived, with malignant transformation leading to complete loss of p27 immunoreactivity. Corticotrophs are quite different to other pituitary cell types in terms of p27 immunoreactivity because both normal and tumorous corticotrophs have low p27 staining, and we speculate that this may relate to their inherent control mechanisms. (*J Clin Endocrinol Metab* **84:** 3823–3830, 1999)

PITUITARY adenomas (PAs) are a common form of endocrine neoplasia causing clinical problems resulting from syndromes of hormone hypersecretion, hypofunction of the residual normal pituitary gland, and mass effects from the tumor bulk itself. Pituitary tumors are usually well-differentiated (1) and may express their appropriate mature pituitary hormone product, although the histogenesis of apparently nonfunctioning PAs (NFPAs) remains unclear. The molecular pathogenesis of pituitary tumors is as yet undetermined other than in a minority of cases; either activation of an oncogene or inactivation of a tumor-suppressor gene has been postulated, but the only common abnormality has been the finding of the *gsp* mutation in 30–40% of somatotroph adenomas (2, 3). However, there is evidence in favor of *ras, hst*, or *nm-23* involvement in the pathogenesis of the more aggressive phenotypes (3, 4).

The cell cycle is controlled at different stages by altered activities of specific cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs) (5, 6). Cyclins activate the CDKs with which they associate and then direct the activated kinase complexes to appropriate substrates, for example, the retinoblastoma protein (7). The resulting phosphorylation causes these substrates to execute key steps in cell proliferation; thus, retinoblastoma is inactivated by phosphorylation allowing cell cycle progression. The activity of cyclin-CDK complexes is regulated by two families of CDKI proteins that generally inhibit cell cycle progression. The INK4 group includes p16, p18, p15, and p19; p15 and p16 abnormalities have been described in various human malignancies (8). The kip/cip family comprises three structurally related proteins: p21, p27, and p57 are all capable of

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binding to and inhibiting most cyclin/CDK complexes, including cyclin D and cyclin E complexes (9–12). Overexpression of these CDKIs causes cell cycle arrest, and thus, these agents act as negative regulators of the cell cycle. The p27 gene maps to chromosome band 12p13 (13, 14) and regulates progression from G_1 into S phase by binding to and inhibiting the cyclin E/CDK and cyclin D/CDK complexes for entry into S phase (9, 12). p27 is present in large amounts in quiescent cells and declines when cells proliferate in response to mitogenic signals, such as growth factors and cytokines (15–17). In proliferating cells, p27 is expressed at a threshold level, much of it bound in a complex with cyclin D/CDK4 (9, 18, 19). As quiescent cells enter the cycle, the level of cyclin D complexes exceeds that of p27, countering its inhibitory effects. The levels of p27 in relation to cyclin D/CDK4 are crucial in determining whether the latter is enzymatically active or not (20).

It has been reported that mice with a genomic knockout of the p27 gene develop multiorgan hyperplasia, resulting in increased animal size, abnormalities in the thymus, retina, adrenal glands and gonadal organs, and pituitary tumors that develop from the pars intermedia and immunostain for ACTH (21–23). Recently, it has been shown that mice haploinsufficient for the p27 gene also show increased animal size and the spontaneous development of intermediate lobe pituitary tumors, and are sensitive to irradiation- or chemical-induced carcinogenesis (24). It has been suggested that p27 could be important in the genesis of pituitary tumors.

Several groups, including our own, have previously investigated p27 messenger (m) RNA expression and the presence of gene mutations in human corticotroph PAs, concluding that abnormalities of p27 mRNA expression do not seem to be important in the pathogenesis of benign corticotroph tumor formation (25–28). However, our preliminary studies of p27 immunostaining suggested decreased protein expression in pituitary carcinoma, but with no obvious change in benign corticotrophinomas. We have now sought to verify whether the amount of p27 protein product might be involved in the pathogenesis of human pituitary tumors, specifically those with an ACTH-secreting phenotype and aggressive forms of pituitary tumors, using a larger group of tissues and more precise quantitation.

Materials and Methods

Tumor specimens

We studied 107 pituitary tissue samples removed at transsphenoidal surgery. These were classified histologically into 20 normal pituitaries, 73 PAs, 9 aggressive pituitary tumors, and 5 metastatic pituitary tumors. The normal pituitaries were sections cut from tissue removed at transsphenoidal surgery for presumptive tumors that proved on standard hematoxylin and eosin (H and E), immunohistochemical, and reticulin staining to consist of normal pituitary cells and architecture. The normal pituitaries included tissue from patients with the clinical diagnosis of Cushing's disease (n = 14), prolactinoma (n = 3), acromegaly (n = 1), NFPA (n = 1), and arachnoid cyst (n = 1). Abnormal pituitary tissue was classified as adenoma, aggressive adenoma, or carcinoma. The 73 adenomas were categorized as GH-secreting tumors (n = 19), ACTH-secreting tumors (n = 21), prolactinomas (n = 7), 2 TSH-omas, and NFPAs (n = 18); we also looked at FSH-omas (n = 6), all of which showed markedly increased FSH secretion in *in vitro* culture (29) and clinical signs suggestive of increased FSH secretion in 2, although FSH immunostaining did not show uniform FSH positivity in these tumors. Aggressive pituitary tumors showed invasive growth and/or recurrence; this group included five NFPAs, two prolactinomas, and two somatotroph adenomas. The pituitary carcinoma group included three ACTH-secreting tumors and two prolactinomas; these patients had histologically verified extrapituitary metastases. All patients with the clinical diagnosis of Cushing's disease routinely received 6–8 weeks of medical therapy with metyrapone and/or ketoconazole to normalize cortisol levels before surgery.

Immunohistochemistry

Tissue preparation. All tissues were collected at routine transsphenoidal surgery and prepared for pathological examination in a standard manner. Paraffin sections cut at 3 μm were air-dried, then placed in a 60 C oven overnight. Sections were de-waxed in xylene, followed by immersion into a solution of 750 μl 30% hydrogen peroxide and 50 ml methanol for 10 min to block endogenous peroxide. Sections were rehydrated to tap water, ready for antigen retrieval. Sections for p27 immunostaining required heat-mediated antigen retrieval treatment (30). Sections were "superheated" for 4 min in 0.01 M citrate buffer (pH 6.0), then placed into tap water immediately to avoid drying of sections. Sections were transferred to phosphate-buffered saline (PBS) before immunostaining.

Immunostaining–p27. Immunohistochemistry was performed using a standard avidin-biotin complex (ABC) method by an automated staining machine (Optimax Plus 1.5; BioGenex Laboratories, Inc. Finchampstead, Berkshire, UK). The primary antibody, antihuman p27 (31), was placed on sections at 1:50 dilution for 40 min at room temperature in a wet chamber. Sections were washed in PBS and then incubated in a biotinylated anti-mouse second layer for 30 min. Sections were again washed in PBS, then incubated in the avidin-biotin peroxidase complex (Vectastain Elite ABC peroxide kit PK6200; Vector Laboratories, Inc., Peterborough, UK) for 20 min. After three washes in PBS, sections were visualized with activated 3′3′ diaminobenzidene-tetrahydrochloride solution (DAB) (Kentec DAB tablets 4170; Biostat, Stockport, UK) for 10 min; this resulted in a brown end-product. Sections were counterstained with Gill's hematoxylin, dehydrated through graded alcohol before mounting in DPX (BDU, Poole, UK). In the tumor samples, consecutive sections were immunostained for the relevant pituitary hormone to ensure that the area counted for p27 was homogeneous for the hormone stain.

Immunostaining–pituitary hormones. Pituitary hormone stains were performed to establish the hormone phenotype of the tumor samples. GH, ACTH, TSH, LH, and FSH antibodies were supplied by DAKO Corp. (Oxford, UK). The prolactin antibody was purchased from Novacastra (Newcastle-upon-Tyne, UK). Sections were incubated overnight in the primary antibody at 4 C. The standard ABC protocol was followed thereafter. A normal human pituitary was used as a positive control; the hormone antibody was omitted and replaced by mouse immunoglobulin as a negative control.

Double-labeling. Seven normal pituitary samples underwent double-staining for p27 and each of the pituitary hormones (32). Sections were stained for p27 antibody using the avidin-biotin peroxidase method and visualized using DAB. Sections were then stained for pituitary hormones (ACTH, LH, FSH, prolactin, GH, and TSH) using the avidin-biotin alkaline phosphatase conjugate and the Fast Red TR/naphthol AS-MX detection system (product no F4648; Sigma, Dorset, UK). This permits the ratio of p27 expression in normal hormone-secreting cells to be determined. ACTH-, LH-, FSH-, prolactin-, GH-, and TSH-positive cells showed red cytoplasmic staining, whereas p27-positive cells showed brown nuclear staining. Cells that were negative for both stains had clear cytoplasm and blue nuclei.

H and E and reticulin staining. Sections from each pituitary sample were stained with H and E stain as a general stain to demonstrate the various tissue components. A reticulin stain was performed to provide an indication of tissue architecture.

Controls. Specificity of the p27 staining was assessed initially by preabsorption of the antibody with the protein used in its generation; this completely abolished p27 staining.

Positive controls: Human tonsil tissue was used as a positive control because this contains lymphoid tissue with variable proliferative activ-

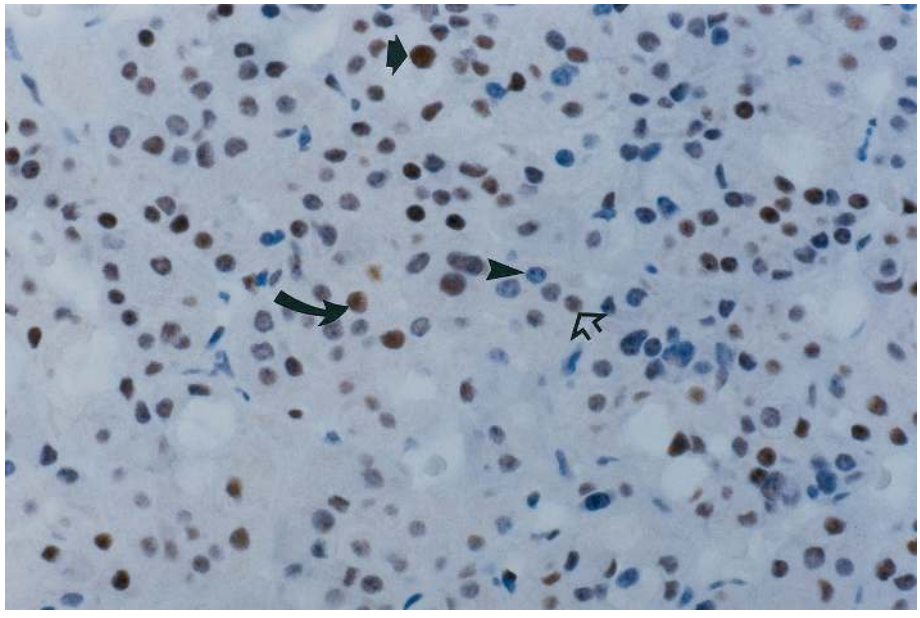

FIG. 1. Immunohistochemistry for p27 in normal pituitary. The *arrowhead* shows a cell nucleus staining negative for p27, the *curved arrow* shows a cell with moderate p27 staining, and the *straight arrow* shows a strongly staining pituitary cell nucleus; these latter two were counted as positive staining. The *open arrow* shows a weakly staining nucleus, which we have counted separately.

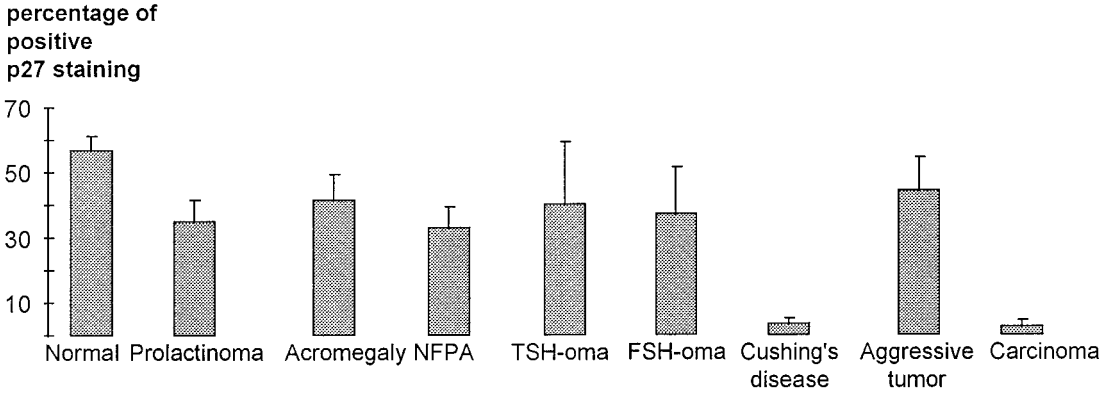

FIG. 2. Percentage of positive p27 immunostaining cells in normal pituitaries (n = 20) and in prolactin- (n = 7), GH- (n = 19), TSH- (n = 2), FSH- (n = 6), and ACTH- (n = 21) secreting tumors, as well as in NFPAs (n = 18) and aggressive (n = 9) and metastatic (n = 5) pituitary tumors. Data are expressed as mean ± SEM.

ity. In the mantle zone the cells are predominately quiescent, demonstrating high levels of p27, whereas cells in the germinal centers are highly proliferative and express low levels of p27.

Negative controls: Negative controls were used in each staining batch in which the primary antibody was omitted and replaced by mouse immunoglobulin (negative control mouse immunoglobulin; Biogenex). The ABC protocol was followed thereafter. Positive and negative controls were run with every experimental tissue section. All sections were examined at ×40 magnification using bright field microscopy.

Quantitation. Cells were assessed for the intensity of staining, and cells with strong or moderate staining were counted as positive, cells with no staining were counted as negative, whereas cells with weak staining were scored separately (Fig. 1). Sections were assessed by a single observer blinded to the diagnosis. In each section, ~500 cells were analyzed. The percentage of p27-positive or -negative cells was then expressed as a ratio of positive or negative cells to the total number of cells counted. Photographs were taken with a Sony 3CCD Color video camera and printed on Sony color video printer UP-5200MDP (Sony Corp., Japan).

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). A normal pituitary and a pituitary ACTH-secreting carcinoma were analyzed by semiquantitative RT-PCR, as described earlier (25). In short, total RNA was obtained and reverse-transcribed into complementary DNA (cDNA), which underwent duplex PCR with intron-skipping primers for p27 and the houskeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), giving rise to products of 358 and 198 bp, respectively. The PCR products were run on 2% ethidium bromide-stained agarose gels. The absorbance values were measured for each band by densitometry (model DS670 image densitometer; Bio-Rad Laboratories, Inc., Hemel-Hempstead, Hertfordshire, UK), using the Molecular Analyst PC software for Bio-Rad Laboratories, Inc.'s Image Analysis systems, and were expressed as optical density units. A ratio between p27 and GAPDH was obtained for each individual sample.

Statistical Analysis. The data were analyzed by nonparametric tests (Mann-Whitney *U* test and the Kruskal-Wallis test, as appropriate) using the Arcus Quickstat Biomedical version 1.2 (Buchan I; Addison Wesley Longman Ltd., Cambridge, UK).

Results

The immunohistochemical analysis in normal pituitary tissue revealed a mean of 57% positive cells for p27 staining. Analyzing the positively staining cells in normal tissue and in tumorous tissue as a whole, there was a significant difference between these two groups (p27 staining mean ± SEM: normal 57 ± 4%, tumor 28 ± 3%; $P < 0.001$ by Mann-Whitney *U* test; Fig. 2). However, the tumorous group was inhomo-

geneous and showed significant variability in p27 staining ($P < 0.001$ by Kruskal-Wallis test; Fig. 2). The GH-secreting tumors, nonfunctioning tumors, prolactinomas, TSH-omas, FSH-omas, and aggressive tumors showed slightly less p27 staining than normal pituitary tissue; however, corticotroph adenomas and metastatic tumors showed a significantly lower level of p27 expression ($P < 0.0001$ compared with normal tissue; $P < 0.01$ compared with the other tumor types; Figs. 2, 3, and 4). The number of p27-negative cells was also considerably higher in corticotroph and metastatic tumors ($P < 0.01$; Fig. 4).

Using semiquantitative RT-PCR, we analyzed the mRNA expression of an ACTH-secreting pituitary carcinoma and compared it with a normal pituitary (Fig. 5). However, although the pituitary carcinoma studied showed very little p27 immunostaining (78% of the cells negative), its mRNA expression was not proportional to the protein expression: the normal pituitary showed a p27 to GAPDH ratio of 0.31, whereas the carcinoma showed a slightly higher ratio (0.72; Fig. 5).

Normal pituitary tissue was also examined using double-labeling for p27 and each of the pituitary hormones to accurately locate the p27 staining to the different cell types (Figs. 6

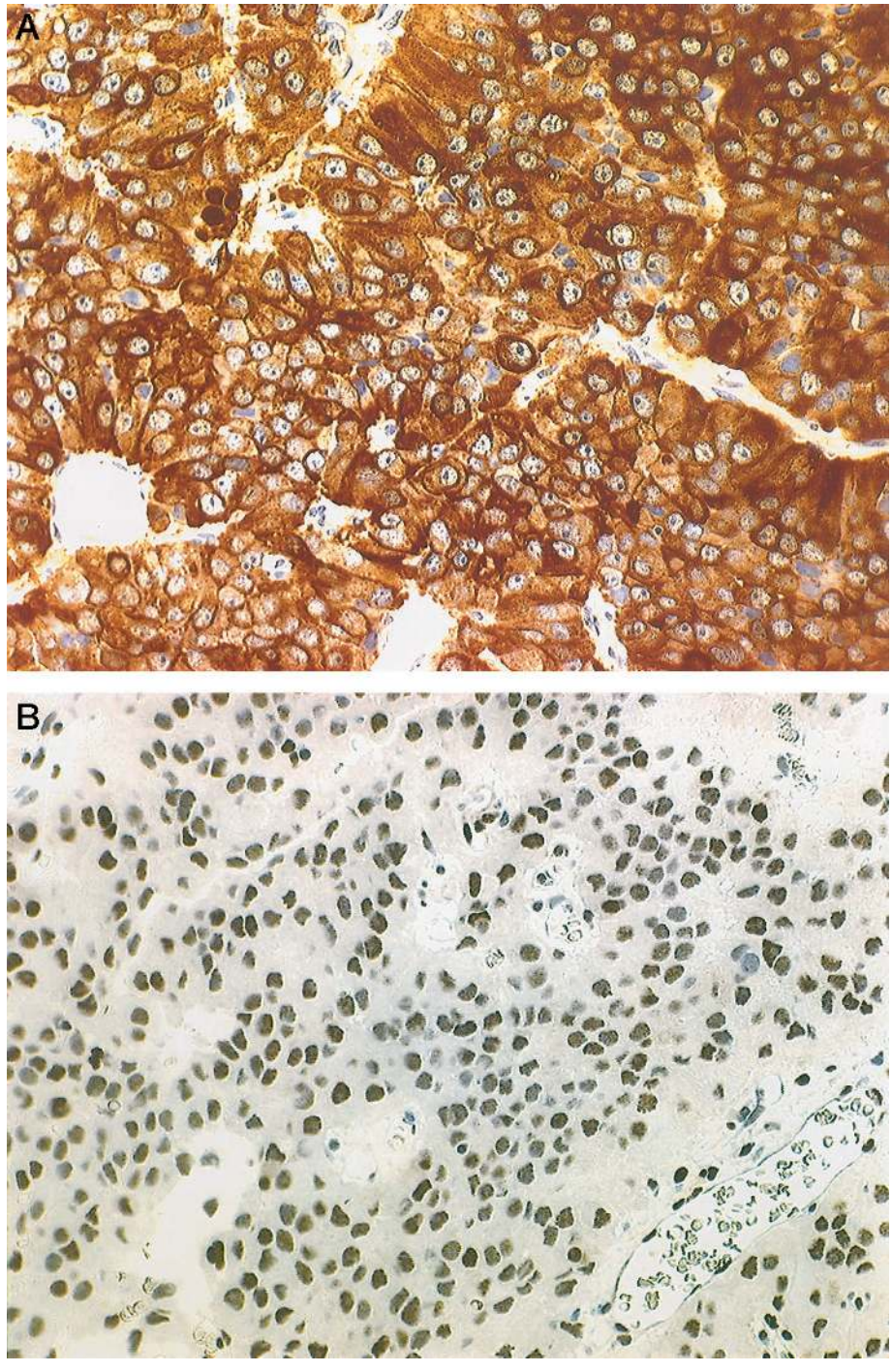

FIG. 3. A, Immunostaining for GH in a somatotroph tumor. B, Immunostaining for p27 in a corresponding area.

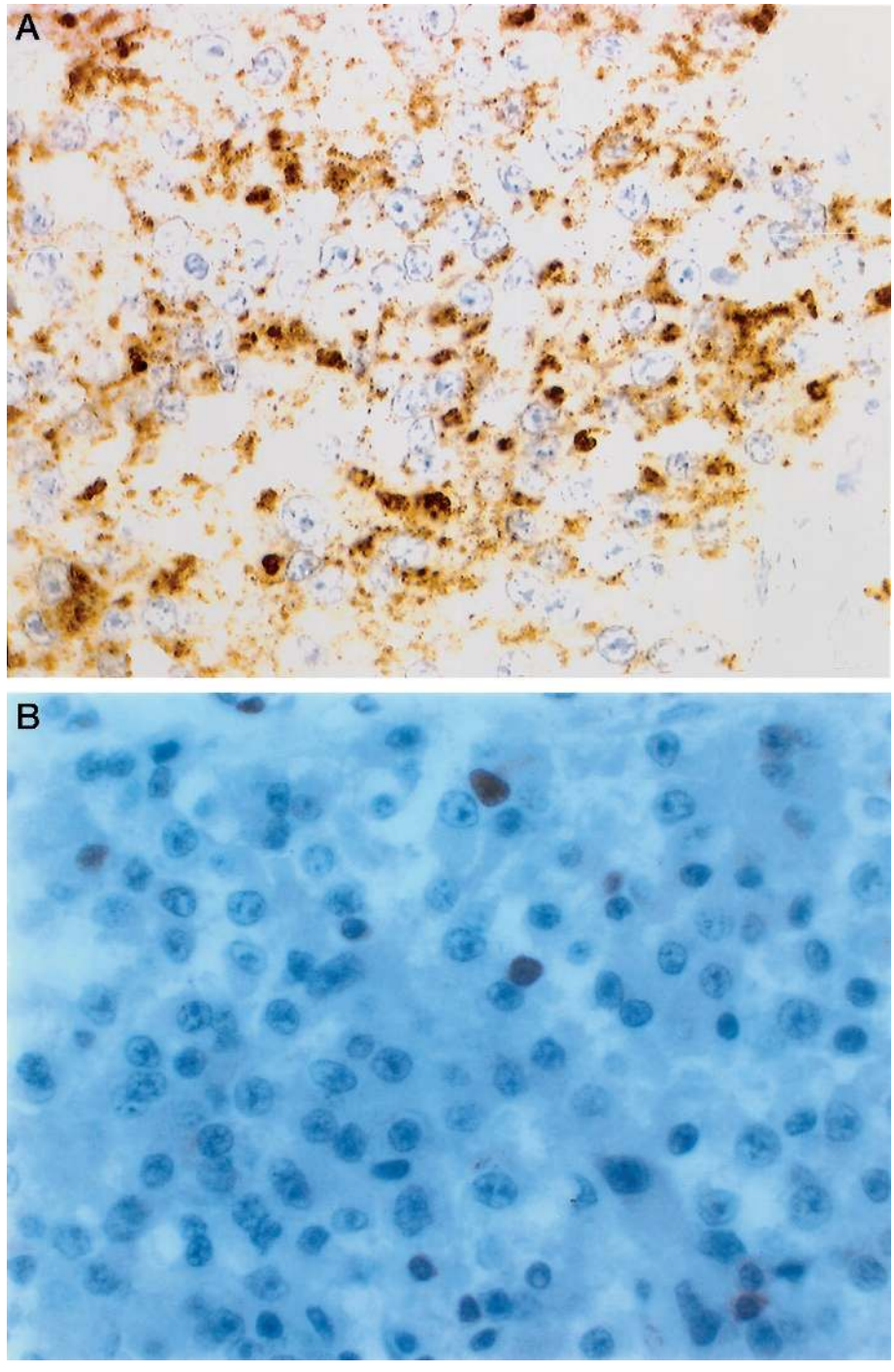

FIG. 4. A metastatic prolactin-secreting pituitary tumor is shown stained for prolactin (A) and for p27 (B).

and 7). Around 40% of the GH-, prolactin-, TSH-, FSH-, and LH-secreting cells showed positive p27 staining, whereas the corticotroph cells uniquely showed a much lower level of p27 expression (3%) that was significantly different to each of the other cell types (Kruskal-Wallis test, $P = 0.006$; individual comparisons, $P < 0.05$). The p27 staining for the normal tissue from patients with the clinical diagnosis of Cushing's disease was similar to those with other pathology (p27 staining in normal tissue from Cushing's disease 57 ± 4% *vs.* normal tissue from other tumors 55 ± 10%; $P = 0.8$).

We compared the p27 expression of each type of hormone-secreting cell in normal pituitary with the p27 expression of PAs secreting the corresponding hormone. In each case, we noted that the p27 expression in a particular cell type in the normal pituitary was higher than in the corresponding tumor [normal GH cells *vs.* somatotrophinoma cells ($P = 0.02$); normal prolactin cells *vs.* prolactinoma cells ($P = 0.026$), normal FSH cells *vs.* FSH-oma cells ($P = 0.01$), normal ACTH cells *vs.* corticotropinoma cells ($P = 0.001$ by Mann-Whitney U test); Fig. 8]. There was a similar difference in the TSH-secreting cells compared with the TSH-omas, although, due to the small sample size, this did not attain statistical significance ($P = 0.1$; Fig. 8).

Discussion

Recent studies have emphasized the importance of p27 protein, a member of the CDKI family, as a regulator of cell cycle progression. In this study, we observed a decrease in the percentage of cells expressing p27 in pituitary tumors compared with normal pituitary tissue; p27 was particularly low in corticotroph adenomas and in metastatic pituitary carcinomas. However, aggressive PAs were not different from benign pituitary tumors in terms of p27 immunostaining. The staining for p27 protein was significantly weaker in normal corticotroph cells compared with other normal pituitary cell types.

There is increasing evidence for an association between cyclin-CDK complexes and CDKIs and cancer (33). There is aberrant expression of p27 in neoplasms with a marked decrease of p27 expression in some benign and malignant neoplasms compared with normal tissue (34). The 12p13 chromosomal band is known to be deleted in leukemias (13), although no mutations of p27 were found in 147 human primary solid tumors (14). In addition, a decrease or absence of p27 protein is a powerful negative prognostic marker in patients with breast (35, 36) and colorectal carcinomas (31, 37). High cyclin E and low p27 expression is associated with increased mortality among breast cancer patients (35). The correlation of p27 expression and the aggressiveness of gastric carcinomas shows that there is a significant difference between high p27 expression and low p27 expression in the survival of patients with gastric carcinomas (38). However, although certain of the CDKIs show frequent mutations in a variety of different malignancies (33, 39), no gene abnormalities were found in 28 PAs in one study (26) or in 18 nonfunctioning and somatotroph tumors in another, apart from the known polymorphism described at codon 109 (27). We were also unable to demonstrate any gene mutation in the exonic sequence of p27 in corticotrophinomas (25). Furthermore, p27 mRNA levels in human PAs were not different from normal tissue in several studies (25, 28), and we were also unable to show any loss of heterozygosity in the p27 region in a subset of corticotrophinomas investigated (25). However, it has been reported that there is less p27 protein expressed in PA tissue when 4 normal human pituitaries (removed at autopsy) were compared with 14 adenomatous pituitaries, with ACTH-secreting adenomas showing the lowest level of positivity (28, 34), although this was not confirmed in a more recent study investigating NFPAs and GH-secreting tumors (27). Our results, using a rigorously quantitative technique on a large number and variety of tumors and normal pituitaries, clearly suggest that adenomatous tissue shows less p27 compared with normal pituitaries. Because the normal pituitary contains a heterogeneous variety of cell types, we also carried out double-labeling studies to compare directly p27 immunopositivity within a given secreting cell, and again we noted a significant

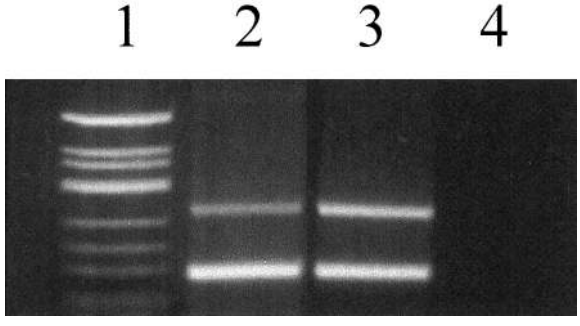

FIG. 5. Products of a duplex PCR using p27 (top band) and a housekeeping gene (GAPDH) were run on 2% agarose gel. *Lane 1*, a size marker (Phi X174 DNA/*Hinf-1*); *Lane 2*, a normal pituitary; *Lane 3*, a pituitary carcinoma secreting ACTH; *Lane 4*, the water control.

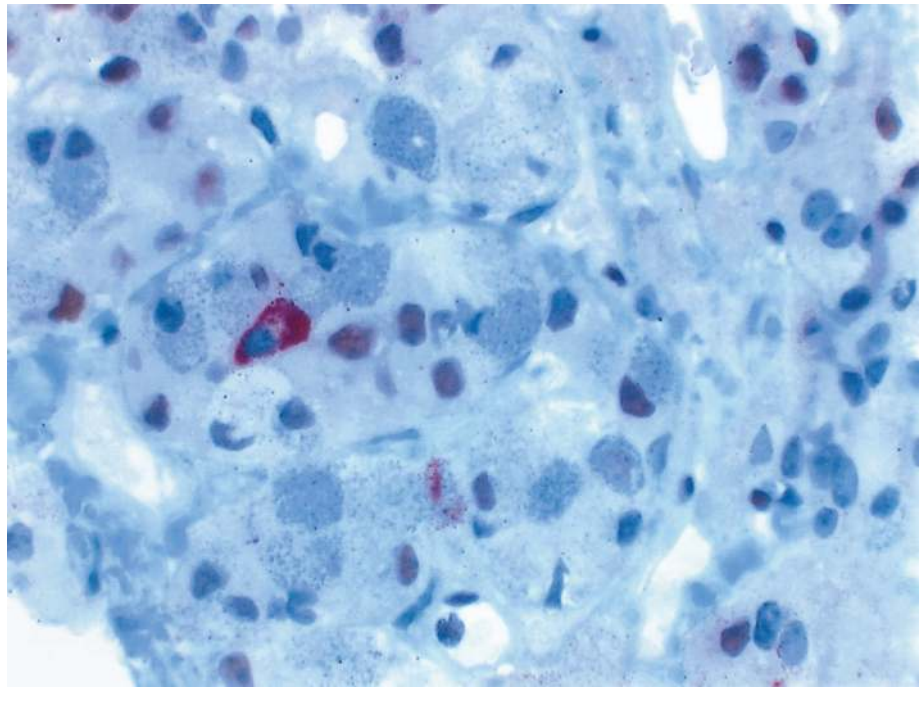

FIG. 6. Double-staining for TSH and p27 in a normal pituitary. TSH is represented by the *red* cytoplasmic stain, whereas p27 shows the *brown* nuclear stain. The TSH-positive cell is negative for p27 (*red* cytoplasm, *pale blue* nucleus).

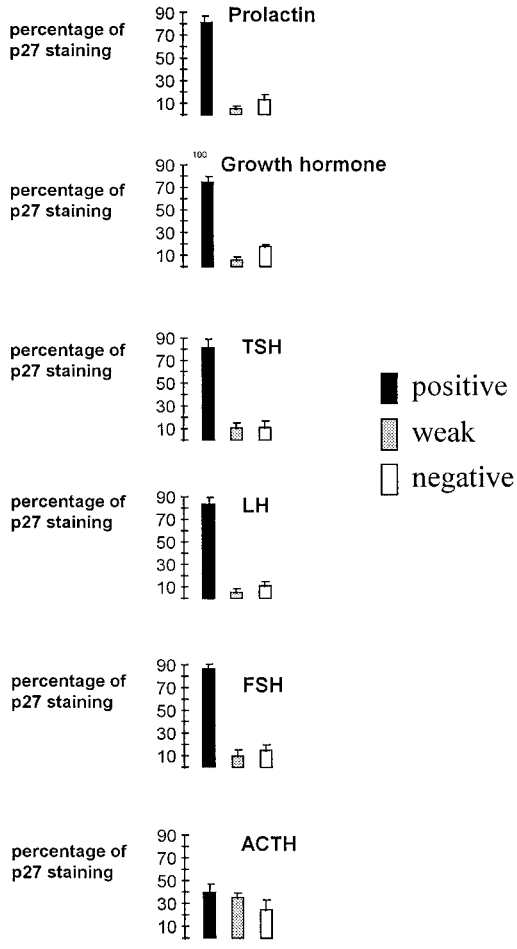

FIG. 7. Double-staining for p27 and prolactin, GH, TSH, LH, FSH, or ACTH in seven normal pituitaries. The data are expressed as percentage of positive, weak, or negative p27 staining cells of all hormone-positive cells; mean ± SEM. Statistical analysis for positive p27 staining showed a significant difference among the different cell types (Kruskal-Wallis test, $P = 0.006$), with individual comparisons showing significant differences between ACTH cells as opposed to GH, prolactin, TSH, FSH, and LH cells ($P < 0.05$), whereas cells in the latter group were not significantly different from each other.

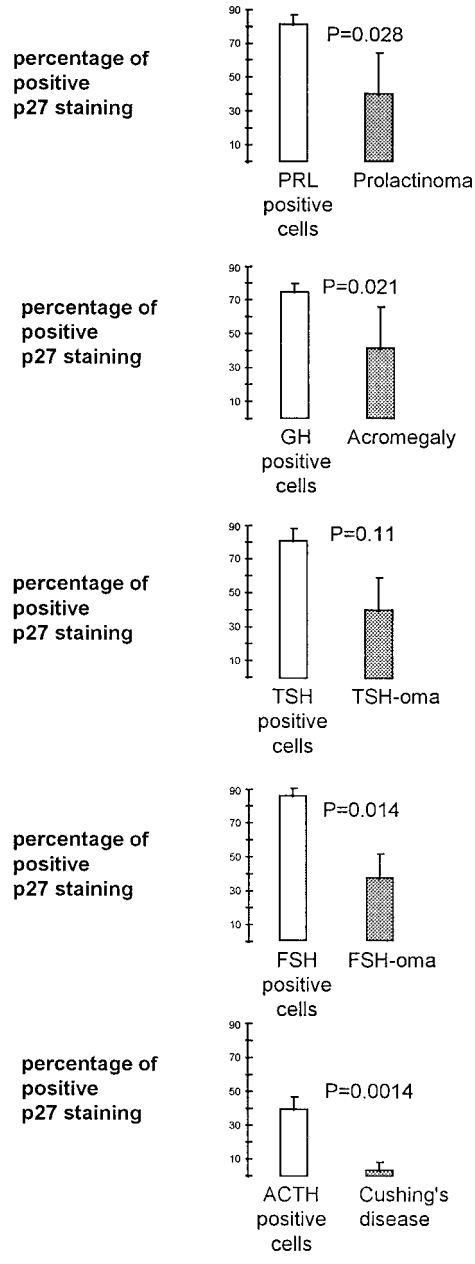

FIG. 8. A comparison of p27 staining between hormone-secreting cells in the normal pituitary (n = 7) and the corresponding hormone-secreting PA cells [prolactin- (n = 7), GH- (n = 19), TSH- (n = 2), FSH- (n = 6) and ACTH- (n = 21) secreting adenomas]. All data are shown as mean ± SEM.

loss of p27 in tumorous cells of any class compared with the untransformed cells.

It is likely that p27 protein expression in PAs and pituitary carcinomas is regulated by translational and posttranslational mechanisms. Indeed, it has been found that p27 is degraded by the ubiquitin-proteasome pathway, that this process has been suggested to be more active in more malignant tumors, and that its activity is inversely correlated with the p27 protein content of the tissue (17, 37). Aggressive colorectal carcinomas show higher specific proteolytic activity and lower levels of p27 (37). Recently, another protein has been implicated in p27 protein regulation: Jab1 (Jun activation domain-binding protein) controls the activity of p27 by facilitating its degradation, possibly by assisting relocalization of p27 from the nucleus into the cytoplasm (40, 41). Unlike other cell cycle proteins, for which correlations have been found between the abundance of these proteins and changes in their mRNA content, a decline in p27 protein occurs in the presence of constant amounts of mRNA and protein synthesis (17). Here, we also demonstrate that a sample with very little p27 protein content showed a comparable amount of mRNA expressed in accordance with earlier more extensive studies of p27 mRNA expression (25, 28). In all types of pituitary tumors, p27 staining was significantly less than in the corresponding normal cell type, in accordance with data from nonendocrine tumors, suggesting that p27 is, indeed, an important regulator of cell proliferation. Finally, although only a few pituitary carcinomas were assessed, our data indicate that the progression from adenoma to carcinoma is also associated with a further loss of p27 protein

expression. This seemed to be true for both ACTH- and prolactin-secreting tumors.

As corticotroph tumors showed a lower level of p27 staining compared with other benign pituitary tumors, we speculated whether this is a phenomenon characteristic of corticotroph adenomas or is present in normal ACTH-producing cells, as well. Previous studies have suggested that in normal rat pituitary more p27 is present in prolactin, gonadotroph, and TSH cells, and less in GH and ACTH cells (42); in normal human pituitaries, gonadotrophs and thyrotrophs show the highest level of p27 staining, whereas ACTH cells show the lowest level (28). Our results suggest that low p27 staining is solely a characteristic of corticotrophs; all other cell types have populations containing ~20 times as many p27-positive cells as ACTH-containing cells (see Fig. 7). This may indicate that corticotroph cells are more proliferative than other cell types, although there are few data in the literature on the proliferative rate of different pituitary cell types (43). However, in humans corticotroph tumors characteristically show increased invasiveness and recurrence compared with other hormone-secreting pituitary tumors (44, 45). It would, therefore, seem that low p27 immunopositivity is a characteristic of corticotrophs that may reflect their intrinsic proliferative rate, although there is no direct evidence in support of this speculation. However, adenomatous transformation of any cell type seems to lead to a reduction in p27 immunopositivity from the preceding basal level specific to that cell.

In conclusion, in the normal pituitary there is variable expression of the CDKI p27, with relatively weaker staining in corticotrophs compared with other cell types. Tumorous transformation is associated with a reduction in p27 nuclear staining in each cell type, with a virtual absence of p27 in pituitary carcinomas. We suggest that these changes may reflect the proliferative activity of the cells and in turn implicate an abnormality of the pathway regulating p27 breakdown in pituitary oncogenesis.

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