

Short Communication

Aberrant p27^{kip1} Expression in Endocrine and Other Tumors

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The p27^{kip1} (p27) gene encodes an inhibitor of cyclin-dependent kinase activity. The expression of p27 protein in normal and neoplastic tissues was investigated by immunoblotting and immunohistochemistry. Immunoblotting studies detected a 27-kd protein band that was decreased in neoplastic pituitary tissues compared with normal pituitary. Immunostaining of 177 tissues showed abundant expression of p27 protein in normal tissues with decreased numbers of immunoreactive cells in adenomas and carcinomas in both endocrine and nonendocrine tissues. p27 expression was inversely related to the proliferation marker Ki-67 antigen detected with monoclonal antibody MIB-1. Parathyroid adenomas and hyperplasias had similar Ki-67 labeling indices; however, hyperplasias had threefold more p27-positive cells than parathyroid adenomas, suggesting that p27 immunostaining may be useful in distinguishing between these two conditions. These results indicate that there is widespread aberrant p27 expression in hyperplastic tissues and in benign and malignant neoplasms compared with normal tissues. Immunohistochemical analysis of p27 along with Ki-67 may be used to assess the biological behavior of various neoplasms, to classify hyperplastic and neoplastic tissues, and to study cell cycle regulation during tumor progression. (Am J Pathol 1997, 150:401–407)

Cell proliferation is regulated by many factors. Recent studies have shown that cyclins and cyclin-dependent kinase (CDK) complexes have important regulatory roles during cell cycle progression.^{1–4}

The activity of cyclin-CDK complexes is regulated by two families of proteins that generally inhibit cell cycle progression. These proteins belong to two large groups based on their structural and functional properties. The INK4 group includes p16, p15, p18, and p19, which are proteins with four ankyrin repeats. They form complexes with CDK4 and/or CDK6 and the D-type cyclins and have functional activities dependent on the presence of a normal retinoblastoma protein.^{5,6} Maximal expression of this group occurs during the middle of S phase in proliferating cells. A high frequency of gene deletions of p15 and p16 and mutations of p16 have been observed in human tumors and cell lines, suggesting that these genes may function as tumor suppressors.^{7–9}

The other group of CDK inhibitors includes p21/WAF1/CIP1, p27/kip1 and p57/kip2,^{10–16} which are proteins that inhibit kinase activities of preactivated G₁ cyclin E-CDK2, cyclin D-CDK4/6, and other cyclins.^{10–16} Overexpression of kip proteins causes cell cycle arrest. p27 protein has a 42% amino acid homology with p21 and a 47% homology with p57 protein at the amino-terminal domain that mediates inhibition of CDK. The kip proteins all have a nuclear localization signal in their carboxyl-terminal domain. The role of kip protein in regulating cell cycle progression in normal and neoplastic cells has not been elucidated. Recent studies with p27-deficient mice

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that developed multiple organ hyperplasia indicate that this CDK inhibitor has anti-proliferative activity *in vivo*.¹⁷⁻¹⁹

We used immunochemical studies to analyze the distribution of p27 in normal, hyperplastic, and neoplastic endocrine and other tissues to investigate the role of p27 in controlling cell cycle progression and tumorigenesis.

Materials and Methods

Formalin-fixed, paraffin-embedded tissue blocks from 177 patients who had surgery at the Mayo Clinic between 1980 and 1995 were used.

Immunoblot Analysis

Proteins from thyroid and pituitary tissues were extracted in the presence of protease inhibitors as previously reported,^{20,21} and one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with a 12% gel using the discontinuous buffer system of Laemmli.²² The electrophoresed proteins (50 μ g) were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Richmond, CA) and subjected to immunoblot analysis with anti-p27 antibody (Transduction Laboratory, Lexington, KY) at a 1/2000 dilution. The reaction product was detected by enhanced chemiluminescence (ECL) using a kit from Amersham Life Science (Arlington Heights, IL). HeLa cells were used as positive controls for immunoblotting. After blotting with anti-p27, the blots were reprobed with anti- β -actin (Sigma Chemical Co., St. Louis, MO) at a 1/2000 dilution to check for equal loading of the gel. The ratio of normal tissues to tumors was determined after ECL by scanning densitometry with a CS9000U densitometer (Shimadzu Corp., Tokyo, Japan).

Immunohistochemistry

Sections for immunostaining were cut at 5 μ m. Antibodies included anti-p27 (Transduction Laboratory) used at a 1/1000 dilution and MIB-1 (AMAC, Westbrook, ME) used at a 1/50 dilution. Sections were treated with 0.1 mol/L citrate, pH 6.0, in an 800-W microwave oven for 15 minutes for antigen retrieval before immunostaining. Immunostaining was done with the Elite avidin biotin peroxidase kit (Vector Laboratories, Burlingame, CA) used according to the manufacturer's specifications. Slides were counterstained with hematoxylin for 1 second. Positive controls for immunostaining consisted of tonsil tissues that were positive for both p27 and Ki-67. Negative

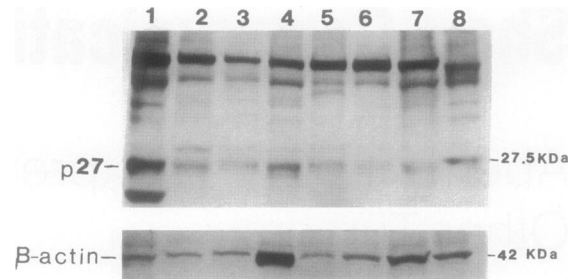


Figure 1. Immunoblot analysis of normal and neoplastic tissues. Top panel: Immunoblotting with p27 antibody. Lane 1, normal pituitary; lanes 2 and 3, pituitary adenoma; lane 4, normal thyroid; lane 5, papillary carcinoma; lane 6, follicular carcinoma; lane 7, anaplastic thyroid carcinoma; lane 8, positive control HeLa cells. Fifty micrograms of protein was used for lanes 1 through 7 and twenty micrograms of protein was used for the HeLa cell positive control. Bottom panel: Immunoblotting of the same samples with β -actin antibody to check for equal loading of the gel.

controls consisted of substituting normal mouse serum for the primary antibodies.

Quantitation

The percentage of cells expressing p27 and Ki-67 was determined by counting 1000 cells per slide. Results were expressed as the mean \pm SEM. Statistical differences were analyzed using the Student's *t*-test.

Results

Immunoblotting

Immunoblotting analysis detected a 27-kd band in all of the tissues examined (Figure 1). Normal pituitary expressed two- to eightfold more p27 compared with pituitary adenomas. Normal thyroid had the same or lower amounts of p27 as some thyroid neoplasms when β -actin was used to check for equal loading. However, the immunohistochemical staining showed abundant colloid protein in the normal thyroid, which probably diluted the p27 protein used for immunoblotting. Additional bands of higher molecular sizes were noted in all samples, but these were of uncertain significance. A lower molecular weight band of approximately 18 kd was also present in the normal pituitary and may be due to protein degradation.

Immunohistochemistry

Immunohistochemical staining revealed nuclear localization of p27 protein in normal and neoplastic tissues. Analysis of normal, benign, and malignant thyroid and parathyroid tissues showed a higher percentage of cells immunostaining for p27

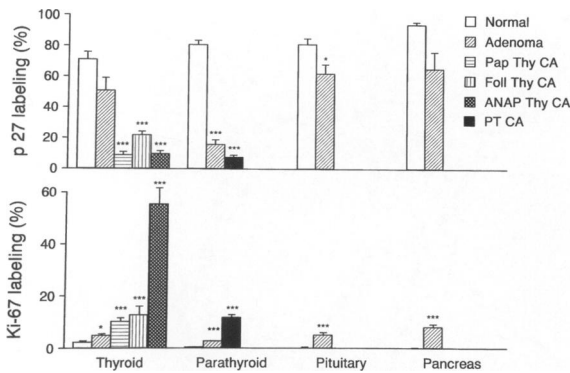


Figure 2. Analysis of p27 and Ki-67 in normal and neoplastic endocrine tumors. Samples were quantified as described in Materials and Methods and results expressed as the percentage of labeled cells (mean \pm SEM). Samples include normal thyroid (n = 6), follicular adenoma (n = 10), papillary carcinoma (Pap Thy CA; n = 10), follicular carcinoma (Foll Thy CA; n = 10), anaplastic carcinoma (ANAP Thy CA; n = 10), normal parathyroid (n = 7), parathyroid adenoma (n = 9), parathyroid carcinoma (PT CA; n = 10), normal pituitary (n = 4), pituitary adenoma (n = 14), normal pancreas (n = 5), and pancreatic tumors (n = 5). Statistical significance was present for most neoplasms compared with the respective normal tissues. $\Psi < 0.05$; $\Psi\Psi < 0.01$; $\Psi\Psi\Psi < 0.001$ compared with normal tissues.

in normal tissues compared with neoplasms (Figures 2 and 3). Thyroid and parathyroid adenomas generally had more cells positive for p27 compared with carcinomas. Similar results were observed for normal pituitary and pancreatic islets compared with pituitary adenomas and islet cell tumors (Figure 2). Parathyroid hyperplasias had threefold more cells positive for p27 compared with parathyroid adenomas (Table 1).

Analysis of other neuroendocrine tumors also showed a higher percentage of normal cells positive for p27 compared with the adjacent tumors (Table

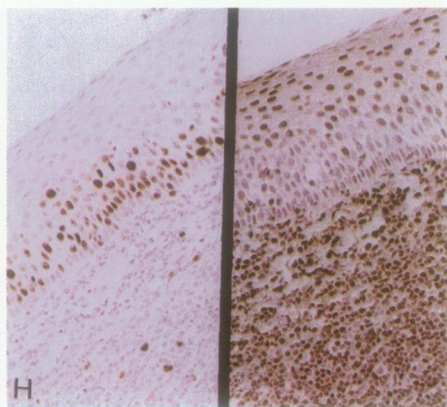
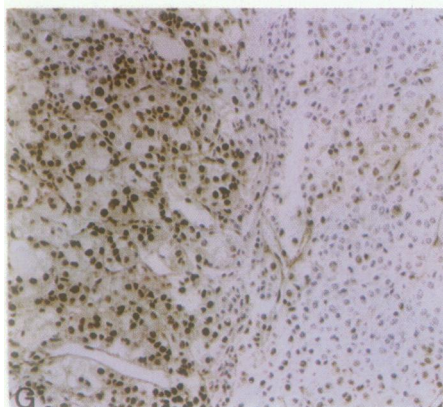
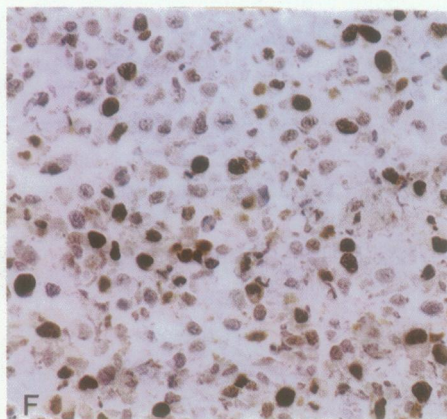
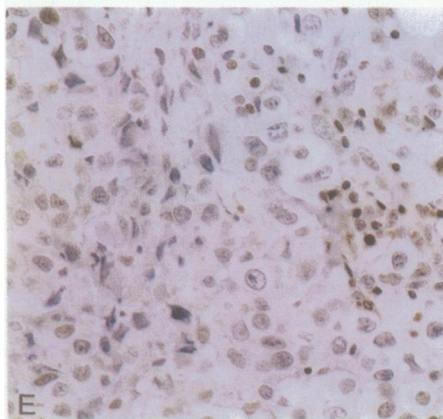
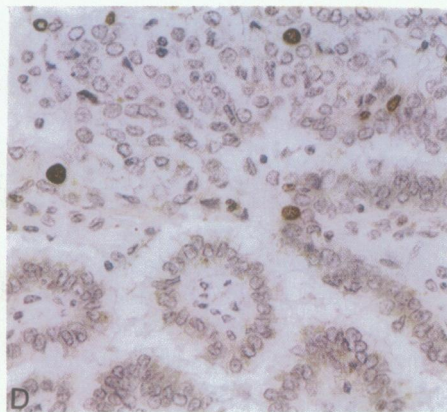
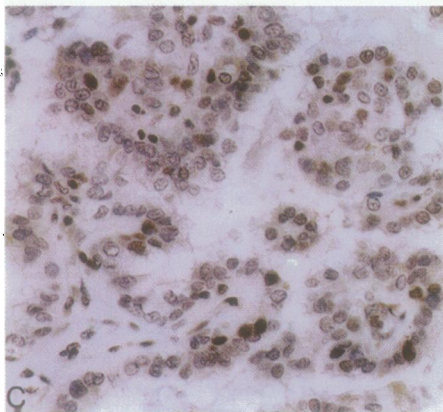
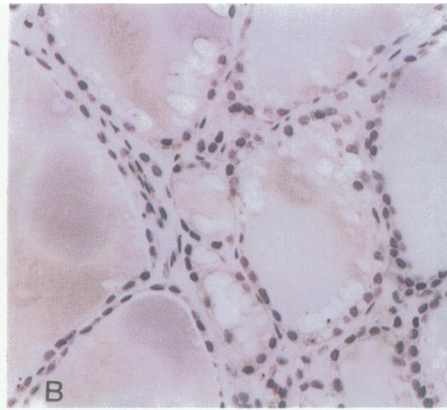
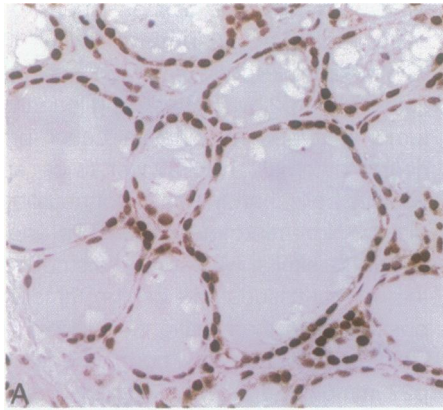
1). Some endocrine tissues, such as adrenal medulla, had fewer p27-positive cells (43%) compared with the adrenal cortex in which most cells in all three zones were p27 positive. Ganglioneuroblastomas, a tumor that generally has a better prognosis than neuroblastomas, had almost twice as many p27-positive cells than the cases of neuroblastomas (Table 1). Non-neuroendocrine cells and tumors also showed a similar widespread distribution of p27 with the normal tissues and benign tumors having more positive cells than malignant neoplasms (Table 1). Unexpectedly, very few normal hepatocytes showed p27 immunoreactivity, whereas hepatocellular carcinomas had many p27-positive cells (Table 1). Normal colonic crypts adjacent to the colonic adenocarcinomas had a low p27 labeling index (2.8 ± 0.8 in four cases), probably reflecting the high turnover rate of these cells. Adenocarcinomas from various sites, including prostate and lungs, had fewer p27-positive cells than the adjacent normal tissues. Normal lymphocytes from lymph nodes and tonsils had a high percentage of p27-immunoreactive cells (Figure 3), whereas lymphomas had lower numbers of cells positive for p27 (Table 1). Mesenchymal cells, including fibroblasts, endothelial cells, and adipocytes, were mostly positive for p27.

Ki-67 immunostaining of normal and neoplastic tissues generally showed an inverse pattern of nuclear immunoreactivity compared with p27; ie, normal tissues that were strongly positive for p27 generally had fewer MIB-1-positive cells, whereas highly malignant tumors, such as anaplastic thyroid carcinomas, had few p27-positive cells but many MIB-1-

Table 1. p27 and Ki67 Immunoreactivity in Normal and Neoplastic Tissue

Tissue	Diagnosis	n	% positive cells	
			p27	Ki67
Adrenal	Normal medulla	4	43 \pm 4.5	0.2 \pm 0.1
Adrenal	Pheochromocytoma	4	33 \pm 1.7	12 \pm 5.6
Adrenal	Ganglioneuroblastoma	3	76 \pm 6	7.3 \pm 3
Adrenal	Neuroblastoma	5	40 \pm 12	66 \pm 9.5
Breast	Normal	4	76 \pm 7	1.2 \pm 0.5
Breast	Adenocarcinoma	6	46 \pm 8.7	24 \pm 4
Colon	Adenocarcinoma	7	2.9 \pm 0.3	43 \pm 7.6
Liver	Hepatocellular carcinoma	3	39 \pm 13	65 \pm 19
Lung	Non-small-cell carcinoma	7	35 \pm 5	39 \pm 8
Lung	Small-cell carcinoma	9	17 \pm 3.6	79 \pm 2.4
Lymph node	Lymphoma	2	53 \pm 31	60 \pm 32
Parathyroid	Normal	7	81 \pm 3.4	0.3 \pm 0.1
Parathyroid	Hyperplasia	7	51 \pm 6.5	2 \pm 0.3
Parathyroid	Adenoma	9	16 \pm 3.2	2.6 \pm 0.2
Parathyroid	Carcinoma	10	7.3 \pm 1.8	11.7 \pm 1.1
Prostate	Adenocarcinoma	2	50 \pm 9.5	13 \pm 5.5
Thyroid	Medullary thyroid carcinoma	3	32 \pm 6.8	5.3 \pm 1.2
Skin	Merkel cell carcinoma	6	49 \pm 8.8	69 \pm 5.7
Skin	Melanoma	5	13 \pm 5.9	57 \pm 13

The percentage of positive cells, or labeling index, was obtained by randomly enumerating 1000 cells per slide.



positive cells (Figures 2 and 3 and Table 1). Negative control slides did not show staining when the primary antibody was omitted.

Discussion

Recent studies of cell cycle regulation have shown that p27 protein, which belongs to the family of CDK inhibitors, has an important regulatory role in cell cycle progression.¹²⁻¹⁴

In the present study, we observed a marked decrease in the percentage of cells expressing p27 in benign and malignant neoplasms compared with normal tissues. Interestingly, p27 expression was the inverse of Ki-67 antigen, a marker of cell proliferation in many endocrine and nonendocrine tissues. The generation of transgenic knockout mice deficient in p27 has shown that p27 protein inhibits proliferation in some tissues such as the thymus, pituitary, and spleen, leading to hyperplasias of these tissues and an increase in general body weight despite normal levels of serum growth hormone and of insulin-like growth factor-1 when p27 was absent.¹⁷⁻¹⁹

The possible role of p27 as a tumor suppressor gene has been analyzed by various investigators recently.²³⁻²⁷ Analysis of over 500 tumors to date has shown only a few mutations,^{23-25,27} including point mutations in 2 of 36 primary breast carcinomas. This suggests that mutations in p27, although uncommon, may be important for tumorigenesis. The report by Chen et al,²⁶ in which an astrocytoma cell line was transfected with p27 resulting in decreased malignant potential, supports our observations of decreased p27 immunostaining in highly malignant tumors such as anaplastic thyroid carcinomas compared with normal thyroid and benign adenomas and suggests that loss of p27 expression may be associated with tumor progression.

The factors regulating p27 during cell cycle progression are still being investigated. Earlier reports with Mv1Lu mink epithelial cells indicated that cyclin D-CDK4 complexes bound and down-regulated p27 activity, which then reversed CDK2 inhibition enabling G₁ progression.^{12,13} Other studies have shown that *in vivo* transforming growth factor- β down-regulated p27 levels.^{12-14,21,28} Poon et al²⁹

observed that p27 was elevated in cells arrested in G₀ by growth factor deprivation or contact inhibition. They noted that cyclin D1-CDK4 acted as a reservoir for p27 and that p27 was redistributed from cyclin D1-CDK4 to cyclin A-CDK2 complexes during S phase or when cells were arrested by growth factor deprivation, lovastatin treatment, or ultraviolet irradiation. However, the role of various factors, including transforming growth factor- β , and contact inhibition *in vivo* may not be mediated by p27 *in vivo*, as p27 knockout mice still had intact cell cycle arrest mediated by transforming growth factor- β and contact inhibition.¹⁷

The Ki-67 antigen detected with MIB-1 antibody is expressed in all stages of the cell cycle except G₀.³⁰ Some investigators have suggested that Ki-67 may not be essential for cell proliferation.^{31,32} However, detection of Ki-67 with antibodies such as MIB-1 has been widely used to assess tumor behavior in histopathology.³³ Recent studies have shown that Ki-67 antibodies recognize nuclear proteins of 345 and 395 kd.³⁴ The inverse relationship between p27 and Ki-67 immunostaining was striking for normal tissues and carcinomas in this study and suggests that both of these markers may be useful in assessing the biological potential of various neoplasms. An unexpected observation in this study was that, although parathyroid adenomas and hyperplasias had similar Ki-67 labeling indices, there was threefold more p27-positive cells in hyperplasias compared with adenomas. Because it may be difficult to distinguish between parathyroid adenomas and hyperplasias, p27 immunostaining may be useful in assisting to make this distinction.

The widespread immunoreactivity of anti-p27 antibody in formalin-fixed paraffin-embedded tissues indicates that it can be used to study the role of p27 in normal, hyperplastic, and neoplastic cells and in tumor progression. With the *in situ* approach used in our analysis, one can readily distinguish immunoreactive stromal cells from hyperplastic and neoplastic parenchymal cells, which often exhibited decreased p27 immunoreactivity compared with the stromal cells. Based on the distribution of p27 nuclear immunoreactivity in various pathological states, this cell cycle protein may be more useful in separating hy-

Figure 3. Immunohistochemical staining for p27 and Ki-67 in normal and neoplastic tissue. **A:** Normal thyroid showing many cells positive for p27. **B:** Normal thyroid with most cells negative for Ki-67. **C:** Papillary thyroid carcinoma stained for p27. **D:** Papillary carcinoma stained for Ki-67. **E:** Anaplastic thyroid carcinoma with most cells negative for p27. The benign inflammatory cells in the stroma show positive nuclear staining. **F:** Staining of the same anaplastic carcinoma for Ki-67 shows many positive cells, indicating a high rate of proliferation. **G:** Pituitary adenoma and normal pituitary. Most of the normal pituitary cells on the left are positive for p27 whereas the adenoma cells on the right have decreased staining for p27. **H:** Normal tonsil showing Ki-67 staining in the proliferating keratinocytes at the base of the squamous mucosa on the left, but there is none in most lymphoid cells. Most of the lymphoid cells on the right are positive for p27. The nonproliferating cells in the squamous mucosa are mostly positive for p27. Magnification, $\times 300$.

perplastic and adenomatous from normal endocrine tissues such as in parathyroid diseases and less useful in distinguishing between adenomas and low-grade carcinomas. Because expression of other members of these two families of cell-cycle-inhibitory proteins such as p16 is also decreased in various neoplasms, including breast carcinomas³⁵ and pituitary adenomas,³⁶ additional studies should provide more insights into the role of these cell-cycle-inhibitory proteins in tumor development and progression.

In summary, there is aberrant expression of p27 in neoplasms with a marked decrease of p27 expression in benign and malignant neoplasms compared with normal tissues. The decreased expression of p27 in these neoplasms suggests that, with increased proliferation, the amount of immunoreactive p27 protein per cell decreases. The exact role of p27 abnormalities in tumor development is uncertain. As mutations are relatively uncommon in the p27 gene, other mechanisms, such as translational control with decreased p27 protein³⁷ or down-regulation of p27 by specific mitogens³⁸ or possibly by brain-specific activators such as the noncyclin activator p35,³⁹ may occur during tumor development. Immunostaining for p27 along with Ki-67 may be used to assess the biological behavior of various neoplasms and to study cell cycle regulation during tumor progression.

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