Review

p27^{kip1}: A Multifunctional Cyclin-Dependent Kinase Inhibitor with Prognostic Significance in Human Cancers

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p27^{kip1} (p27) is a member of the universal cyclin-dependent kinase inhibitor (CDKI) family. p27 expression is regulated by cell contact inhibition and by specific growth factors, such as transforming growth factor (TGF)- β . Since the cloning of the p27 gene in 1994, a host of other functions have been associated with this cell cycle protein. In addition to its role as a CDKI, p27 is a putative tumor suppressor gene, regulator of drug resistance in solid tumors, and promoter of apoptosis; acts as a safeguard against inflammatory injury; and has a role in cell differentiation. The level of p27 protein expression decreases during tumor development and progression in some epithelial, lymphoid, and endocrine tissues. This decrease occurs mainly at the post-translational level with protein degradation by the ubiquitin-proteasome pathway. A large number of studies have characterized p27 as an independent prognostic factor in various human cancers, including breast, colon, and prostate adenocarcinomas. Here we review the role of p27 in the regulation of the cell cycle and other cell functions and as a diagnostic and prognostic marker in human neoplasms. We also review studies indicating the increasingly important roles of p27, other CDKIs, and cyclins in endocrine cell hyperplasia and tumor development. (*Am J Pathol* 1999, 154:313–323)

Recent studies have shown that cyclins and cyclin-dependent kinase (CDK) complexes have important regulatory roles during cell cycle progression^{1–7} (Figure 1). Cyclin-CDK complexes are in turn regulated by the cyclin-dependent kinase inhibitors (CDKIs), which generally inhibit cell cycle progression (Table 1). These proteins fall into two families based on their structural and

functional properties. The INK4 group includes p16/INK4A (p16), p15/INK4B (p15), p18/INK4C (p18), and p19/INK4D (p19). They all have four ankyrin repeats and form complexes with CDK4 and/or CDK6 and the D-type cyclins. They have functional activities that are dependent on the presence of a normal retinoblastoma protein.^{8–10} Maximal expression of the INK4 proteins occurs during the middle of the S phase in proliferating cells. Both p15 and p16 show a high frequency of gene deletions, and various human tumors and cell lines have mutations of the p16 gene, suggesting that these genes may function as tumor suppressors.^{11–13}

The second group of CDK inhibitors, the Cip/Kip family, includes p21/WAF1/CIP1 (p21), p27/kip1 (p27) and p57/kip2 (p57).^{14–22} These proteins inhibit kinase activities of pre-activated G₁ cyclin E-CDK2, cyclin D-CDK4/6, and other cyclins. The Cip/Kip proteins are designated as universal CDKIs because they interact with various CDK complexes, with cyclins A, E, D1, D2, and D3, and CDKs.¹⁵ Overexpression of the kip proteins leads to cell cycle arrest. Members of the kip proteins share a great deal of homology. p27 protein has a 42% amino acid homology with p21 and a 47% homology with p57 at the amino-terminal domain, the region that mediates inhibition of CDK. Kip proteins all have a nuclear localization signal at their carboxyl-terminal domain. Unlike the INK4 group, which inhibits CDK4/6 only, the Cip/Kip inhibitors can also target CDK2 in complexes.

The p27 gene is located on chromosome 12p13 at the junction of 12p12–12p13.1.²¹ This gene was cloned by several groups in 1994.^{14,16,23} Structural analysis of the p27 protein was recently reported.²⁴ Examination of the crystal structure of the 69-amino-acid amino-terminal inhibitory domain of p27 bound to the phosphorylated cyclin A-CDK2 showed that p27 binding causes large conformational changes in and around the catalytic cleft of

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Figure 1. Schematic view of mammalian cyclin-dependent kinase (CDK) inhibitors and cyclin-CDK complexes in the cell cycle. Members of the INK4 group (p15/INK4B, p16/INK4A, p18/INK4C, and p19/INK4D) and the Cip/Kip group (p21, Waf1/Cip1, p27/Kip1, and p57/Kip2) have inhibitory roles in G₁ to S progression.

Table 1. Members of the Cyclin-Dependent Kinase Inhibitors of the INK4 and Cip/Kip Families

Family	Chromosome Location
INK4	
p15 (INK4B)	9 p21
p16 (INK4A)	9 p21
p18 (INK4C)	1 p32
p19 (INK4D)	19 p13
Cip/Kip	
p21 Waf1/Cip1	6 p21
p27 Kip1	12 p13
p57 Kip2	11 p15

CDK2²⁴ and that p27 has separate binding sites on the cyclin and CDK subunits. This explains how p27 and other Kip/Cip inhibitors can bind isolated subunits.^{15,24} Binding of the p27 cyclin-CDK complex is significantly tighter than binding to the isolated CDK and cyclin subunits, which is consistent with cooperative binding of the two subunits.

p27 was first identified in cells treated with transforming growth factor (TGF)- β or by stimulation of contact inhibition where p27 was found as an inactive form bound to CDK2-cyclin E.^{18,25} The protein was purified from a cyclin E-CDK2 affinity column and characterized by its strong inhibitory activity toward cyclin E-CDK2. p27 can directly inhibit the enzymatic activity of CDK-cyclin complexes and arrest cells in G₁.¹⁸ The association of p27 with CDK-4 cyclin D or with CDK2-cyclin E complexes blocks phosphorylation of CDK4 on Thr 172 and CDK2 on Thr 160 via a CDK activation kinase.^{22,26} p27 can be induced by cyclic AMP and other negative regulators of the cell cycle²² and can be down-regulated by interleukin 2.²⁶

The levels of p27 protein are increased in quiescent cells and rapidly decrease after stimulation with mitogens. Constitutive expression of p27 in cultured cells causes cell cycle arrest in the G₁ phase.^{15,16} When murine BALB/c-3T3 fibroblasts are deprived of serum mito-

Figure 2. Effect of p27 on cyclin E-CDK2 complex. p27 binds to cyclin E-CDK2 complexes and prevent its activation. Although some p27 is present in proliferating cells, it is sequestered and unavailable to interact with cyclin E-CDK2. p27 can be regulated by the same enzyme it targets for inhibition by becoming a cyclin E-CDK2 substrate, leading to its phosphorylation and proteolysis by the ubiquitin-proteasome pathway. Phosphorylation of retinoblastoma (Rb) protein leads to G₁ to S progression.

gens, p27 accumulates in these cells.²⁷ This finding was correlated with inactivation of G₁-cyclin-CDK complexes and with cell cycle arrest in G₁. Inhibition of p27 expression with antisense oligonucleotides prevents cell cycle arrest in response to mitogen depletion, indicating that p27 is an essential component of the pathway that connects mitogenic signals to the cell cycle.²⁷

Although p27 inhibits cyclin E-CDK2, recent studies have also shown that p27 can serve as a substrate for cyclin E-CDK2.²⁷ Using a murine fibroblast model, it was shown that cyclin E-CDK2 can directly phosphorylate p27 (Figure 2), and the cyclin E-CDK2-dependent phosphorylation of p27 results in elimination of p27 from the cell, allowing transition from G₁ to S phase.²⁸

Other investigators demonstrated Ras-mediated down-regulation of p27 that involves suppression of synthesis leading to an increase in the degradation of the p27 protein.²⁹ It is postulated that Ras function is required in late G₁ for down-regulation of p27 and passage of the cell through the restriction point.²⁹

The role of TGF- β in regulating p27 has been investigated in many systems.^{18,30-32} Using a C3H 10T1/2 mouse fibroblast model it was shown that cyclin E-CDK2 inhibits p27 in the growth-arrested state and that TGF- β down-regulates the steady-state level of the p27 protein.³⁰ Mal et al³¹ showed that mink lung epithelial cells arrested in G₁ by TGF- β could be rescued from this arrest by disabling of p27 via adenovirus oncoprotein E1A. Qian et al demonstrated that TGF- β down-regulates p27 protein and mRNA levels in cultured rat anterior pituitary cells.³²

Functions of p27

There are many putative functions attributed to p27 (Table 2). Extensive investigations have been performed to elucidate the role of p27 as a CDKI in normal and neoplastic cells.^{14,16,18,27-30} Some studies suggest a putative role as a tumor suppressor gene. Loss of p27 protein

Table 2. Putative Functions of p27^{Kip1}

"Universal" cyclin-dependent kinase inhibitor that regulates progression through the cell cycle. ^{1-7,14,16,18}
Potential tumor suppressor gene. ⁴⁴⁻⁴⁷
Promoter of apoptosis. ^{37,38}
Regulator of drug resistance in solid tumors. ³⁵
Role in cell differentiation in muscle, oligodendrocytes, osteoblasts, and granulosa cells. ³⁹⁻⁴²
Safeguard against inflammatory injury. ⁴³

expression may result in tumor development and/or progression; however, this loss of expression does not appear to result from gene mutations.³³⁻³⁶ More than 500 tumors have been examined for p27 mutations, and less than 5 of these have shown specific mutations.³⁴ These have included a stop codon at position 76 of an adult T-cell leukemia and hemizygous deletion of the p27 gene in a B-cell non-Hodgkin's lymphoma³³ in addition to 2 point mutations in an analysis of 36 primary breast carcinomas.³⁴ One of the mutations in the breast carcinomas was a polymorphous mutation at codon 142 and the other a nonsense mutation at codon 104.³⁴

St. Croix et al³⁵ reported that p27 has a role in regulating drug resistance in solid tumors. Human and mouse tumor cells grown as multicellular spheroids in three-dimensional culture show a consistent up-regulation of p27 (up to 15-fold).³⁵ When a mammary tumor cell line (EMT-6) was treated with antisense p27 oligonucleotides, there was increased cell proliferation, with restoration of the drug- or radiation-induced cell cycle perturbations that were repressed in spheroid culture.³⁵

Recent studies have implicated p27 as a promoter of apoptosis.^{37,38} Using the MDA-MB-231 breast carcinoma cell line to overexpress p27, Katayose et al³⁷ found that apoptosis was increased when measured by several techniques.³⁷ Similar results were observed in other cell lines. Interestingly, p27 did not induce apoptosis in the total cell population. As cells in G₁ did not undergo apoptosis, a possible explanation for this observation was that the cells arrested in G₁ may be protected in some way from induction of apoptosis.³⁷

Many studies have shown that p27 has a role in regulating differentiation in some tissues.³⁹⁻⁴² In a study of mouse embryo skeletal muscle, functional assays showed that ectopic p27 expression enhances the efficiency of MyoD-initiated muscle differentiation. It was proposed that p27 acts as a trigger for CDK1 while myoblasts are exiting the cell cycle and initiating differentiation. In a study using oligodendrocyte cell precursors from rats, Durand et al⁴⁰ showed that p27 accumulates progressively in the precursor cells as they proliferate and that p27 is present at high levels in mature oligodendrocytes, implicating p27 accumulation in the differentiation of oligodendroglial cells.

In a study of the effects of CDKIs on the regulation of the G₁ phase cyclin-dependent kinases, it was noted that parathyroid hormone increases p27 levels but not p21 levels in osteoblasts. The data implicate parathyroid hormone in blocking entry of cells into the S phase and inhibiting cell proliferation when p27 accumulates within these cells. The effect of parathyroid hormone is medi-

ated through the protein kinase A pathway.⁴¹ Robker and Richards reported that follicle-stimulating hormone and estradiol regulate granulosa cell proliferation during the development of preovulatory follicles by increasing the levels of cyclin D2 relative to p27, whereas luteinizing hormone terminates follicular growth by down-regulating cyclin D2 while up-regulating p27 as well as p21.⁴² Thus, the LH surge with high cyclic AMP levels induces the granulosa cells to enter a nonproliferative or more differentiated stage as they enter the luteal phase.

p27 has been implicated in the protection of some cells against inflammatory injury.⁴³ Using mice with an engineered deletion of the p27 gene,⁴⁴⁻⁴⁶ a model of experimental glomerulonephritis was used to analyze immune-mediated inflammation. Renal function decreases in p27 null mice compared with controls with wild-type p27, and this is associated with increased glomerular cell proliferation, apoptosis, and matrix protein accumulation.⁴³ Both tubular epithelial cell proliferation and apoptosis are increased in p27 null mice after ureteral obstruction. The authors concluded that p27 may have a general role in protecting cells and tissues from inflammatory injury. Interestingly, this *in vivo* effect of p27 on apoptosis in p27 null mice is the opposite effect observed *in vitro* where p27 enhanced apoptosis.³⁸

There are two lines of evidence indicating that p27 suppresses cell proliferation *in vivo*. Malignant human brain tumor cell transfection with the p27 gene leads to inhibition of proliferation and cell cycle arrest in G₁.⁴⁷ Ectopic overexpression of p27 is associated with a striking decrease in aneuploid cells, loss of anchorage-independent growth in soft agar, and failure to induce tumor development in a xenograft model. Mice lacking the p27 gene show an increase in body weight, thymic hypertrophy, and hyperplasia of pituitary intermediate lobe adrenocorticotrophic hormone cells, adrenal glands, and gonadal organs.⁴⁴⁻⁴⁶ Analysis of CDK2 in the thymocytes of these knockout mice show a 10-fold increase in the activity of this enzyme. A surprising finding was that the effects of TGF- β , rapamycin, and contact inhibition on cell proliferation remained unchanged in p27 null mice, indicating that the presence of p27 is not an absolute requirement for this pathway.⁴⁴

Several recent studies have implicated p27 in inhibition of cell cycle progression by homophilic cell-cell interaction and in tumor metastasis.^{48,49} St. Croix et al examined the role of the homophilic cell-cell adhesion molecule E-cadherin in contact-dependent growth inhibition using a mouse mammary carcinoma cell line, EMT 16, in a multicellular spheroid model *in vitro*. They observed that E-cadherin expression after transfection with an E-cadherin expression vector resulted in an increase in the level of p27 and showed that E-cadherin was a major growth suppressor as well as an invasion suppressor.⁴⁸ Studies of p27 protein expression in primary colorectal carcinomas and their metastatic foci showed a marked reduction in p27 expression in the metachronous metastases compared with the corresponding primary tumor, suggesting that down-regulation of p27 in circulating tumor cells may confer the ability to grow in an environ-

ment of altered extracellular matrix or intercellular adhesion properties that may facilitate tumor metastasis.⁴⁹

Regulation of p27 Expression

p27 protein levels increase in cells treated with cyclic AMP, lovastatin, rapamycin, and tamoxifen,^{22,23,50,51} and this increase is probably related to the G₁ block produced by these agents. Cells undergoing differentiation also have increased levels of p27 protein.^{52,53} Several studies have shown that the human papilloma virus can regulate p27 activity.³¹ Some studies have shown that p27 levels are regulated by alterations of protein stability; thus, the half-life of p27 is much longer in quiescent cells compared with proliferating cells.⁵⁰

Ubiquitination is the principal mechanism regulating p27 protein degradation.^{54–57} Ubiquitin is a small protein of 7000 MW that is covalently linked to a target protein.^{58–63} The ubiquitin-target protein complex is specified by the ubiquitinating enzymes E1, E2, and E3. The ubiquitin-activating enzymes (E1s) are the first enzymes involved in protein ubiquitination. These enzymes form a thioester bond between the carboxy terminus of ubiquitin and an internal cysteine residue. E2s are designated as ubiquitin-conjugating enzymes, or UbcS, and form a thioester bond between the internal systemic residue and the carboxy terminus of a molecule of ubiquitin. E2 transfers the ubiquitin to ϵ -amino groups of lysine in the target protein. The ubiquitin ligases (E3s) are not as well characterized. They act as substrate recognition factors. The proteasome, which is a multimeric protein complex, recognizes the covalent adduction between ubiquitin and the target protein such as p27, which leads to degradation of the target protein with recycling of ubiquitin.

Pagano et al⁵⁴ showed that the peptide-aldehyde *N*-acetyl-leucyl-leucyl-norleucinal-H (LLnL), an inhibitor of the chymotryptic site on the protease, leads to the accumulation of p27 protein and its ubiquitinated forms of approximating *M_r* 70,000 and *M_r* 100,000, indicating that p27 was polyubiquitinated *in vivo*. Our laboratory has also observed ubiquitin-proteasome regulation of p27 protein in a human pituitary cell line (Figure 3).

When purified p27 was incubated with Ubc2 or Ubc3, a mono-ubiquitinated form of p27 was generated, suggesting that polyubiquitinated p27 requires additional factors such as E3. Extracts from proliferating or S phase cells contain more p27 ubiquitinating and degradation activities than extracts from quiescent cells.^{61,63} Half-life studies have shown that p27 in proliferating cells is six-fold less stable than in quiescent cells,⁵⁴ which explains why p27 is expressed at much higher levels in quiescent cells than in proliferating cells and highlights the translational control of p27 expression by ubiquitin-mediated degradation.

Phosphorylation appears to be an important mechanism for p27 degradation.^{55,56,64} p27 phosphorylation is cell cycle dependent and peaks during the late G₁ phase. The amount of p27 protein is inversely correlated with its phosphorylation. Using human fibroblasts, Monsaki et al⁵⁸ reported that cyclin E/CDK2 phosphorylated

Figure 3. Analysis of the effect of inhibition of the chymotryptic site of the ubiquitin-proteasome pathway on cellular p27. The HP75 pituitary cell line (produced in our laboratory) was treated with the peptide-aldehyde *N*-acetyl-leucyl-leucyl-norleucinal-H (LLnL), an inhibitor of the chymotryptic site on the proteasome, or with the cysteine protease inhibitor *L*-trans-exposuccinic acid (E64) as a control for 16 hours in culture. The cells were homogenized and analyzed for p27 by Western blotting using a monoclonal antibody (Transduction Laboratories, Lexington, KY) and enhanced chemiluminescence (Amersham, Arlington Heights, IL). The samples include the following: **lane 1**, control cells with culture media only; **lane 2**, cells treated with dimethylsulfoxide (100- μ L volume equivalent to the LLnL and E64 vehicle volume); **lane 3**, 50 μ M/L LLnL; **lane 4**, 100 μ M/L LLnL; **lane 5**, 50 μ M/L E64; **lane 6**, 100 μ M/L E64; **lane 7**, HeLa cells used as a p27-positive control. LLnL, but not E64, increased p27 protein in the HP75 cells. β -Actin was used to normalize for protein loading. The graph on the right was generated by densitometric analysis of the film. These results indicate that the ubiquitin-proteasome pathway is one of the mechanisms regulating the expression of p27 protein in pituitary tumor cells.

p27 on threonine 187 *in vitro*, and phosphorylation of p27 affected the stability of the p27 protein. It has been shown that p27 must be phosphorylated by CDK2 on a conserved carboxyl-terminal CDK target site to be degraded by the proteasome.⁵⁶

The importance of phosphorylation and the ubiquitin ligase complex in the degradation of cell cycle proteins such as p27 is derived in part from experiments in yeast.⁶⁵ A specific ubiquitin ligase complex, SCF^{Cdc4P}, when mixed with the ubiquitin-activating enzymes E1, the ubiquitin-conjugating enzyme E2, Cdc34p, and ubiquitin led to the reconstitution of ubiquitination of the phosphorylated Cdk inhibitor Sic1p, providing a molecular basis for the G₁/S transition, which may be a general mechanism for ubiquitination after phosphorylation in eukaryotes.⁶⁵

Although protein degradation by the ubiquitin-proteasome pathway is the principal method of proteolysis of p27, recent studies have shown that methylation may be another mechanism regulating p27 expression. Using various pituitary tumor cell lines, Qian et al⁶⁶ showed that GH₃ and GHRH-CL1 cell lines, both of which produce prolactin and growth hormone, express very little p27 protein or mRNA.³³ Analysis of exon I and part of exon II of the p27 gene using bisulfite genomic sequencing showed that both GH₃ and GHRHCL1 cell lines are hypermethylated whereas the control rat pituitary p27 gene is hypomethylated.⁶⁶ By changing the methylation status of the p27 gene with 5-aza-2'-deoxycytidine they were able to show that methylation and methyltransferase activity regulated expression of p27 in some pituitary cell lines. The importance of methylation of the p27 gene in regulating its function is not known.

Figure 4. Immunohistochemical staining for p27 expression in normal and neoplastic tissues. **A:** Lymph node tissue with p27 staining on left showing strong immunoreactivity in quiescent cells and less staining in proliferating cells. The germinal center (GC) with proliferating cells had few cells positive for p27. Staining with a Ki67 antibody MIB-1, which recognizes proliferating cells, showed the opposite pattern of staining compared with p27 with strong staining of most cells in the GC. Magnification, $\times 200$. **B:** There is selective localization of p27 in normal anterior pituitary cells. Immunostaining of normal anterior pituitary for p27 (nuclear purple staining) and for thyroid stimulating hormone (TSH; brown cytoplasmic staining) shows localization of p27 in some TSH cells (arrows). Magnification, $\times 300$. **C:** Normal and neoplastic breast tissue with strong staining for p27 in normal mammary ducts whereas the invasive carcinoma cells are mostly negative. Magnification, $\times 300$. **D:** Normal and neoplastic prostate tissue showing strong staining for p27 in normal prostatic ducts while the invasive carcinoma cells are weakly positive or negative. Magnification, $\times 300$. **E:** Parathyroid tissue showing strong nuclear staining for p27 in the normal cells on the left whereas the adenoma on the right stains weakly, indicating low expression of p27. Magnification, $\times 250$. **F:** The normal thyroid on the left shows strong nuclear staining for p27 in the follicular cells. The papillary carcinoma on the right shows low expression of p27 protein. Magnification, $\times 250$.

p27 as a Diagnostic and Prognostic Marker

During the past 2 years a large number of studies have examined the diagnostic and prognostic significance of p27 expression in various tumors. Almost all studies report decreased p27 expression in more aggressive tu-

mors⁶⁷⁻⁹⁰ (Figure 4). p27 expression is reported to be an independent prognostic factor or potentially useful in the diagnosis of a broad spectrum of tumors. According to Steeg and Abrams,⁶⁷ for a new prognostic marker to enter into routine clinical use at least three criteria must be met. 1) The marker provides information independent

Table 3. Role of p27 as a Diagnostic/Prognostic Marker in Human Cancers

Type of tumor	Role of p27	Reference
Breast carcinoma	Decreased p27 in carcinoma and it is associated with tumor progression.	Catzavelos et al ⁶⁸
Breast carcinoma	Decreased p27 contributes to tumor progression.	Porter et al ⁶⁹
Breast carcinoma	Lack of p27 associated with poor prognosis.	Tan et al ⁷⁰
Breast and colon carcinoma	Inverse correlation of p27 and tumor malignancy.	Fredersford et al ⁷¹
Colon carcinoma	p27 is an independent prognostic marker.	Loda et al ⁷²
Colon carcinoma	Significant correlation between p27 and tumor grade.	Ciaporone et al ⁷³
Esophageal Barretts-associated adenocarcinoma	Loss of p27 confers poor prognosis.	Singh et al ⁷⁴
Gastric carcinoma	Correlation of p27 and tumor aggressiveness.	Mori et al ⁷⁵
Lung non-small-cell carcinoma	p27 is a prognostic factor, correlates with survival.	Esposito et al ⁷⁶
Lung non-small-cell carcinoma	p27 decreased in carcinoma compared with non-neoplastic lung tissues.	Kawana et al ⁷⁷
Lung non-small-cell carcinoma	p27 is a prognostic factor for survival.	Yatabe et al ⁷⁸
Prostate adenocarcinoma	Low p27 is an independent prediction of treatment failure.	Tsihlias et al ⁷⁹
Prostate adenocarcinoma	Absent or low p27 is an adverse prognostic factor.	Yang et al ⁸⁰
Prostate adenocarcinoma	Low p27 correlates with lymph node metastasis and higher Gleason scores.	Chevillat et al ⁸¹
Prostate adenocarcinoma	Prostate carcinoma with low p27 more biologically aggressive.	Cordon-Cardo et al ⁸²
Malignant melanoma	Loss of p27 is a prognostic indicator of early relapse.	Florenes et al ⁸³
Oral cavity carcinoma	Low p27 association with oral dysplasia and carcinoma.	Jordan et al ⁸⁴
Endocrine tumors	Low p27 associated with higher-grade tumors.	Lloyd et al ⁸⁵
Thyroid tumors	Low p27 associated with more aggressive tumors.	Erickson et al ⁸⁶
Parathyroid tumors	Low p27 associated with carcinoma.	Erickson et al ⁸⁸
Pituitary tumors	Decreased p27 with tumor progression.	Jin et al ⁸⁹
Pituitary corticotroph tumors	Low p27 associated with more aggressive ACTH tumors.	Dahia et al ⁹⁰
Lymphomas	Low p27 in tumors with a higher growth fraction.	Sanchez-Beato et al ⁹³
Lymphomas	p27 expression inversely related to proliferation rate in all lymphomas, except mantle cell type.	Quintanilla-Martinez et al ⁹⁴

of and better than conventional pathological criteria. 2) The marker provides information that can alter treatment decisions. 3) Studies with the marker are reproducible.

Many reports have validated the utility of p27 as a prognostic and/or diagnostic marker (Table 3).

Studies of breast carcinoma⁶⁸⁻⁷⁰ showed that p27 protein expression is lower in more aggressive tumors. The studies used a single immunohistochemical assay for the protein, indicating the reliability of this technique. Catzavelos et al⁶⁸ showed that p27 is a predictor of reduced disease-free survival by Kaplan-Meier analysis. Porter et al⁶⁹ combined analyses of p27 and cyclin E and showed that both of these cell cycle regulators are prognostic markers of tumor behavior. Tan et al⁷⁰ analyzed p27 expression in breast cancers less than 1 cm in size in 202 patients and found that nodal status and low p27 expression are independent prognostic parameters by both univariate and multivariate analyses. They concluded that p27 identified node-negative patients with small invasive breast carcinomas that were at high risk for tumor progression and therefore might benefit from adjuvant therapy.

Three studies have examined p27 expression in colorectal cancers.⁷¹⁻⁷³ Loda et al⁷² showed that the absence of p27 protein expression was a powerful negative prognostic marker in colorectal carcinomas, particularly in stage II tumors, and suggested that this marker may help in the selection of patients who would benefit from adjuvant therapy. These investigators also showed that carcinomas with low or absent p27 protein showed enhanced proteolytic activity for p27, suggesting that the

low p27 expression resulted from increased protease-mediated degradation rather than from altered gene expression. In another study of p27 in multistage colorectal carcinogenesis, Ciaparrone et al⁷³ found a significant correlation between p27 expression and tumor grade with well and moderately differentiated carcinomas expressing higher p27, while the poorly differentiated carcinomas had significantly lower expression.

Other areas of the gastrointestinal tract have also been studied for p27 expression and tumor behavior. In esophageal adenocarcinomas, low p27 protein correlated with higher histological grade, depth of invasion, presence of lymph node metastasis, and patient survival.⁷⁴ Interestingly, in the study of Singh et al, both cytoplasmic as well as nuclear localization of p27 were associated with decreased patient survival.⁷⁴ p27 has also been found to be an independent prognostic factor for patients with gastric carcinomas.⁷⁵

In a study of p27 expression in non-small-cell lung carcinoma, Esposito et al⁷⁶ showed that p27 was a prognostic factor correlating with patient survival. Kawana et al⁷⁷ also examined a group of non-small-cell carcinomas and showed that the p27 labeling index decreased in carcinomas compared with non-neoplastic lung tissues and was inversely related to the proliferation marker Ki-67. Yatabe et al⁷⁸ also found that p27 was a significant prognostic factor in non-small-cell lung carcinoma; however, in the more aggressive small cell carcinoma, they identified an increased p27 expression when compared with the corresponding normal lung epithelium. This vari-

able expression of p27 in endocrine tumors will be discussed below.

Several studies have analyzed p27 expression in prostate adenocarcinoma.⁷⁹⁻⁸² Tsihlias et al⁷⁹ and Yang et al⁸⁰ showed that low p27 expression was an independent predictor of treatment failure and an independent prognostic factor for disease recurrence. Yang et al⁸¹ suggested that patients with low or absent p27 protein expression may be candidates for novel adjuvant therapies. Cheville et al⁸¹ observed that p27 expression correlated with a higher mean Gleason score, lymph node metastasis, and aneuploid cancers, but it did not correlate with subclinical biochemical failure. Cordon-Cardo et al⁸² observed that primary prostate carcinomas with lower levels of p27 protein were more biologically aggressive. In addition, they observed that p27 protein and mRNA were almost undetectable in both epithelial and stromal cells of benign prostatic hyperplasia (BPH), supporting the concept that BPH is not a precursor to prostate carcinoma.

In the first reported study of p27 expression in malignant melanoma, Florenes et al⁸³ observed that this CDKI was correlated with tumor thickness in nodular melanoma but not in superficial spreading melanomas. Although p27 did not appear to influence overall survival for either subgroup, a complete loss of p27 expression had potential importance as a prognostic indicator of early relapse in patients with nodular melanomas.

Analysis of p27 protein expression in squamous cell lesions of the oral cavity showed that p27 was significantly reduced in oral dysplasias and carcinomas compared with that in normal squamous epithelium.⁸⁴ There is also a significant reduction in p27 protein between low- and high-grade dysplasias, suggesting that changes in p27 expression may be an early change in oral squamous cell carcinogenesis.

Analysis of p27 protein expression in endocrine tumor was first reported by Lloyd et al.⁸⁵ They observed decreased expression of p27 in endocrine adenomas and carcinomas compared with normal tissues. However, the changes are not as striking as identified in some non-endocrine tissues, such as breast, prostate, and colonic carcinomas. For example, in a study of thyroid carcinomas, Erickson et al⁸⁶ found similar levels of p27 in high- and low-grade cancers, ie, anaplastic carcinomas and papillary carcinomas. Similar findings were observed in a comparison of different types of papillary carcinomas by others.⁸⁷ In a large series of parathyroid tumors, there was significantly decreased p27 expression in carcinomas compared with adenomas.⁸⁸ Jin et al⁸⁹ reported that in pituitary tumors, the p27 protein levels decreased during progression from adenomas to carcinomas, but the differences were moderate compared with other non-endocrine tumors. Jin et al⁸⁹ showed that regulation in the pituitary was post-translational as the mRNA levels were similar in normal and tumorous pituitaries. Similar findings were reported in parathyroid tumors by Erickson et al.⁸⁸ In ACTH-secreting pituitary tumors, Dahia et al⁹⁰ reported that corticotroph adenomas express p27 protein, although one carcinoma in their series showed loss of p27 protein expression. Analysis of three other pituitary carcinomas by the same investigators showed two tu-

mors with loss of expression, although one case showed moderate expression.

p27 protein expression has been used as a diagnostic marker in some endocrine tumors. Erickson et al found significant differences in the p27 labeling indices between follicular adenomas and follicular carcinomas.⁸⁶ They suggested that immunostaining for p27 might be useful in distinguishing between these two tumors. Similarly, they reported that p27 immunostaining could be used to distinguish between parathyroid adenomas and carcinomas.⁸⁸

The relationship of decreased levels of p27 protein and tumor progression is variable in different endocrine tumors. Our studies of adrenal cortical and medullary tumors showed only slight differences between normal benign and malignant tumors with respect to p27 protein expression.⁸⁵ Yatabe et al⁷⁸ also reported a higher level of p27 in small-cell neuroendocrine lung carcinomas compared with non-small-cell tumors. As small-cell lung carcinomas have a high proliferation rate, one would predict low levels of p27 protein, as in many tissues there is an inverse relationship between tumor proliferation and p27 expression. However, small-cell carcinoma is known to have genetic defects, such as in the Rb and p53 tumor suppressor genes and over expression of c-myc,⁹¹ that might allow this neoplasm to proliferate despite high p27 levels. Recent reports suggest that c-myc could overcome p27-induced growth arrest by allowing cyclin E-CDK2 to function in the presence of elevated levels of p27.⁹²

Analysis of p27 expression in lymphomas has shown that p27 protein is present in quiescent lymphocytes within lymphoid tissues and peripheral blood.⁹³ Lymphomas with a low proliferative rate are mostly positive, whereas tumors with a higher growth fraction have low p27 protein levels. However, p27 expression in some high-grade, mitotically active tumors were increased, such as Burkitt's lymphoma and large B-cell. These tumors also have mutations in the p53 pathway that might allow the cells to escape the inhibitory effects of p27. Quintanilla-Martinez et al⁹⁴ found that in lymphomas p27 expression was inversely related to cell proliferation. Interestingly, all mantle cell lymphomas lacked p27 protein. They postulated that the uncoupling of p27 protein expression from proliferation rate may be related to the high levels of cyclin D1 found in these mantle cell lymphomas.

Role of CDKIs and Cyclins in Endocrine Cell Hyperplasia and Neoplasia

With a few exceptions, endocrine tumors are usually slow growing. Compared with other types of carcinomas, endocrine tumors, such as papillary and follicular thyroid carcinomas, carcinoid tumors, islet cell carcinomas and parathyroid carcinomas, continue to grow slowly even after metastasizing.⁹⁵ These observations suggest that there is significant inhibitory control of cell proliferation in most endocrine tumors and possibly a greater role of CDKIs in endocrine cell proliferation. In addition to genetic alterations in suppressor genes and onco-

Figure 5. Schematic model showing the influence of p27, other CDKIs and cyclins on the development of endocrine cell hyperplasia and neoplasia. Dysregulation of various CDKIs, including p27, p16, p21, and p57 and of the D-type cyclins have been observed in various proliferating endocrine tissues (see text). Dysregulation of CDKIs and cyclins leading to increased cell proliferation in adult endocrine tissues increases the likelihood of developing genetic alterations resulting in tumor development.

genes,^{96–104} several lines of evidence point to an increasing importance of cell cycle protein dysregulation during the development of endocrine tumors^{104–111} (Figure 5).

Studies of parathyroid tumors showed that the PRAD1 oncogene represented a rearrangement of cyclin D1 with a pericentric inversion on chromosome 11.^{99,100} Point mutations in PRAD1 are not needed for tumorigenesis.¹⁰⁷ Dysregulation of CDKI in parathyroid adenomas and carcinomas has been reported, especially with p27.⁸⁸ Pituitary adenomas and carcinomas were found to have dysregulation of various CDKIs, including p16 and p27. Woloshak et al^{105,106} observed decreased expression of p16 in pituitary adenomas compared with normal pituitaries, and they showed that this decreased expression is due to increased methylation of the p16 gene. Similarly, Jin et al⁸⁹ and Dahia et al⁹⁰ showed dysregulation of p27 during progression from normal pituitary to adenomas and carcinomas. Dahia et al⁹⁰ studied corticotroph adenomas and carcinomas and reported loss of p27 expression in most pituitary carcinomas. Our laboratory has shown that two mechanisms of regulation of p27 occur in endocrine cells and tumors, the ubiquitin-proteasome pathway^{54,57} and p27 gene methylation⁶⁵ with the former being the more common mechanism. Our studies have also shown cyclin D expression in pituitaries with a shift from cyclin D2 in normal pituitary to cyclin D3 in immortalized pituitary tumors such as GH₃.¹⁰⁸

In the adrenal cortex another CDKI, p57^{kip2} (p57), is implicated in adrenal cortical tumorigenesis. Mice lacking p57 develop adrenal cortical hyperplasia and cytomegaly.^{103,104} The levels of p57 mRNA are high in normal adrenal cortex and very low in some adrenal cortical adenomas and carcinomas, indicating decreased expression of this CDKI with tumorigenesis. Although the mechanism of regulation of p57 has not been elucidated

in adrenal cortical tumors, the changes in mRNA indicate that post-translational regulation may not be as important as methylation or other epigenetic changes.¹⁰⁹

Numerous abnormalities in oncogenes and tumor suppressor genes have been identified in thyroid tumors and are implicated in tumor progression.^{101,110} However, dysregulation of CDKIs and cyclins also play important roles in thyroid tumor progression, as p27 protein levels are much higher in thyroid adenomas and normal thyroids compared with carcinomas. The CDKI p21, which is a downstream mediator of p53, has been implicated in thyroid tumorigenesis. Zedenius et al showed that thyroid tumors with p53 mutations have markedly reduced p21 expression.^{111,112} Cyclin D1 overexpression has also been observed in Hurthle cell carcinomas compared to adenomas (L. A. Erickson, L. Jin, J. R. Goellner, L. R. Zukerberg, R. V. Lloyd, unpublished observations).

These data highlight the increasing importance of CDKIs and cyclins in regulating endocrine tumor development and progression and should provide models to study the mechanisms involved in differentiation and tumor development in endocrine tissues.

Future Challenges

Although a great deal of knowledge about the role of p27 in cell cycle progression and tumor development has accumulated, there are still many unanswered questions. Preliminary evidence suggests that c-myc may regulate p27 levels.⁹² Other studies indicate that cyclin E,²⁸ Stat proteins (signal transducer and activator of transcription 6) in lymphoid cells,¹¹³ and cyclin D¹¹⁴ can also regulate p27 levels in some cells. A recent study using cultured astrocytes indicated that multiple CDKIs are necessary to maintain cell cycle progression in this system.¹¹⁵ Some tumors, such as mantle cell lymphomas, overexpress cyclin D1 but have very little p27.⁹⁴ Other studies have shown an interaction of p27 and p21 in some tumors with the cleavage of both p27 and p21 resulting in activation of CDK2, leading to increased apoptosis in some cells.³⁸ More experimental data about the interaction of p27 with other cell cycle regulatory proteins are needed.

The rarity of mutations and other genetic alterations in the p27 gene during tumor development is not consistent with its role as a tumor suppressor gene. Investigations into whether there are mutations or other genetic alterations in the ubiquitin-proteasome system leading to increased degradation of p27 and other cell cycle proteins during tumor progression are needed, and the experimental tools are available to address these questions. The recent observations of increased p27 in several human breast cancer cell lines compared with cell lines from normal mammary epithelial cells were surprising and difficult to explain.¹¹⁶ Additional experiments done by transfecting normal and neoplastic mammary lines with a vector containing p27 showed that the increased expression of p27 was associated with decreased cyclin D1 in the neoplastic MCF7 cell line, but not in the normal cell line, and slightly increased levels of cyclin E protein in both cell lines¹¹⁷ indicated that the role of multiple

interacting CDKs and cyclins in regulating G₁ to S progression and their synchronous dysregulation during tumor development requires additional studies. With the complex interactions of CDKs, CDKs, and cyclins, there is an increased likelihood of alterations of these genes and/or their protein products. Finally, analysis of the roles of specific growth factors, hormones, and other influences on p27, other CDKs, and cyclins should provide new insights into the mechanisms underlying the molecular changes leading to cellular differentiation or tumorigenesis.¹¹⁸

Note Added in Proof

Since submission of this review, two significant studies have been published that provide new insights into the role of p27 in tumorigenesis. Franklin et al¹¹⁹ showed that p27 and p18 mediate two separate pathways to collaboratively suppress pituitary tumorigenesis, possibly by controlling the function of Rb. Fero et al¹²⁰ showed that p27 is haplo-insufficient for tumor suppression, belonging to a new class of tumor suppressor genes.

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