Growth Factors, Cytokines, Cell Cycle Molecules

Complex Regulation of the Cyclin-Dependent Kinase Inhibitor p27^{kip1} in Thyroid Cancer Cells by the PI3K/AKT Pathway

Regulation of p27^{kip1} Expression and Localization

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Functional inactivation of the tumor suppressor p27^{kip1} in human cancer occurs either through loss of expression or through phosphorylation-dependent cytoplasmic sequestration. Here we demonstrate that dysregulation of the PI3K/AKT pathway is important in thyroid carcinogenesis and that p27^{kip1} is a key target of the growth-regulatory activity exerted by this pathway in thyroid cancer cells. Using specific PI3K inhibitors (LY294002, wortmannin, and PTEN) and a dominant active AKT construct (myrAKT), we demonstrated that the PI3K/AKT pathway controlled thyroid cell proliferation by regulating the expression and subcellular localization of p27. Results obtained with phospho-specific antibodies and with transfection of nonphosphorylatable p27^{kip1} mutant constructs demonstrated that PI3K/AKT-dependent regulation of p27^{kip1} mislocalization in thyroid cancer cells occurred via phosphorylation of p27^{kip1} at T157 and T198 (but not at S10 or T187). Finally, we evaluated whether these results were applicable to human tumors. Analysis of 100 thyroid carcinomas indicated that p27^{kip1} phosphorylation at T157/T198 and cytoplasmic mislocalization were preferentially associated with activation of the PI3K/AKT pathway. Thus the PI3K/AKT pathway

and its effector p27^{kip1} play major roles in thyroid carcinogenesis. (Am J Pathol 2005, 166:737–749)

Disruption of cell cycle control is frequent in human cancer.¹ Dysregulation of cell proliferation and failure to suppress tumor growth often result from alterations in the activity of Cdk inhibitors.² Ink4 Cdk inhibitors are lost through deletion, point mutations, and/or promoter methylation in a variety of human neoplasms and are thus true tumor-suppressor genes.³ Differently, the Cip/Kip Cdk inhibitor p27^{kip1} does not fit the classic tumor-suppressor paradigm because mutations in the gene encoding p27^{kip1} are rare.⁴ However, because p27^{kip1} inactivation is fundamental for the development of malignancies, p27^{kip1} has been designated a tumor-suppressor protein.⁵

Two mechanisms govern p27^{kip1} inactivation during human carcinogenesis: loss of protein expression and exclusion from the nuclear compartment.^{6,7} The level of p27^{kip1} is reduced (or even absent) in ~50% of human cancers.^{4,6} Cytoplasmic sequestration of p27^{kip1} is a mechanism whereby cancer cells overcome p27^{kip1}-imposed growth inhibition and has been reported for colon,⁸ esophagus,⁹ thyroid,¹⁰ ovarian,¹¹ and breast carcinomas.^{12–14} Importantly, the loss of p27^{kip1} expression and its presence in the cytoplasm of cancer cells are markers that predict shorter disease-free and/or overall survival in patients affected by different types of cancer.^{6,7}

Loss of p27^{kip1} expression in cancer primarily occurs through sustained protein degradation,^{15–17} a four-step

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process that requires phosphorylation of p27^{kip1} at threonine 187 by cyclin E/cdk,^{18,19} recognition of T187-phosphorylated p27^{kip1} by the ubiquitin ligase SCF^{Skp2}, ubiquitylation, and degradation by the 26S proteasome of T187-phosphorylated p27^{kip1}.^{20–22} Cytoplasmic retention of p27^{kip1} may occur through increased export or reduced import.⁷ Interaction of p27^{kip1} with JAB1/CNS5 or phosphorylation of serine 10 (S10) by the hKIS kinase promotes p27^{kip1} export from the nucleus,^{23,24} whereas phosphorylation of threonine 157 (T157) by the protein kinase B/AKT (AKT) impairs its import.^{12–14}

Whereas loss of p27^{kip1} and its cytoplasmic relocalization in human cancer is well established, the signaling pathways that regulate these processes are primarily obscure. In an attempt to cast light on the signaling pathways that govern loss of p27^{kip1} and its cytoplasmic relocalization in human cancer, we studied thyroid follicular cell neoplasms because in these tumors p27^{kip1} is inactivated by both loss of expression and cytoplasmic sequestration.¹⁰ Furthermore, thyroid cancer is unique in that distinct histological features, malignant potential, and degree of differentiation can arise from a single cell and are associated with specific oncogenic lesions.^{25,26} In particular, papillary thyroid carcinomas (PTCs) are characterized by chromosomal rearrangements that result in the activation of the *RET/PTC* tyrosine kinase receptor (3 to 60% of cases),²⁷ by activating mutations in the gene encoding the serine/threonine kinase *BRAF* (28 to 69% of cases)^{28–30} or by overexpression of the *MET* oncogene.³¹ Follicular thyroid carcinomas (FTCs) are instead characterized by activating point mutations in one of the three *RAS* genes (18 to 52% of cases).³² Alteration of the PI3K/PTEN/AKT pathway, by reduced expression of the dual specificity phosphatase *PTEN*^{33–36} or by hyperexpression of AKT, occurs in both FTCs and PTCs.^{37,38}

Proliferative signaling elicited by activated tyrosine kinase receptors or by *RAS* proteins is funneled through a network regulated by the phosphatidylinositol 3' kinase (PI3K) and by the mitogen-activated protein kinase (MAP kinase).³⁹ Activation of PI3K and generation of phosphatidylinositol 3,4,5-triphosphate are required for activation of AKT and p70^{S6K}.⁴⁰ In turn, AKT regulates cell division by increasing cyclin D1 stability⁴¹ and/or by inactivating p27^{kip1} either by regulating p27^{kip1} mRNA expression^{40,42} or by controlling the subcellular localization of p27^{kip1}.^{12–14} Here we report that the PI3K/AKT pathway is activated in ~50% of thyroid carcinomas, and that this signaling cascade contributes to inactivation of p27^{kip1} through AKT-dependent cytoplasmic sequestration of p27^{kip1}.

Materials and Methods

Cell Lines and Reagents

Five human thyroid carcinoma cell lines were used in this study: TPC-1 and NPA (derived from PTCs), WRO (derived from FTCs), and FRO and FB1 (derived from anaplastic carcinomas).^{10,34} All cell lines were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. LY294002 and cycloheximide were from Sig-

ma-Aldrich (St. Louis, MO). Wortmannin was from Calbiochem (Merck KGaA, Darmstadt, Germany).

Tissue Specimens

Thyroid carcinomas were collected from the Surgery B section of the National Cancer Institute "Fondazione G. Pascale" (Naples, Italy) or retrieved from the files of the Department of Functional and Biomorphological Sciences at the University of Naples Federico II. Diagnosis was based on standard histological criteria. We selected paraffin blocks that were free of oxyphilic (Hurtle) changes, which are sources of aspecific cytoplasmic staining, and that included both the tumor and the rim of normal thyroid tissue around it; the latter serving as control of immunohistochemical staining.

Immunostaining

Serial sections were stained using monoclonal anti-p27^{kip1} antibody (dilution 1:4000; Transduction Laboratories, Lexington, KY) and the polyclonal antibody anti-phospho-AKT (Ser473) from Cell Signaling Technology (Beverly, MA) at a dilution of 1:250. After incubation with primary antibodies, the sections were incubated with biotinylated anti-mouse/rabbit immunoglobulins, and with peroxidase-labeled streptavidin (LSAB-DAKO, Glostrup, Denmark). Diaminobenzidine was used to visualize the signal. Sections incubated without the specific antibody and sections incubated with unrelated antibodies served as controls of the technique. Antibody specificity was assessed by competition with antigens used for antibody production (full-length p27^{kip1} and phospho-AKT-blocking peptides, respectively). Tumors were scored as p27^{kip1}-positive or p27^{kip1}-negative depending on a staining cutoff of 50%, as described elsewhere.¹⁰ p27^{kip1} expression was evaluated from both nuclear and cytoplasmic staining. p27^{kip1}-positive tumors with cytoplasmic plus nuclear staining or with only cytoplasmic staining were designated "cytoplasmic" and tumors with only nuclear staining were designated "nuclear." If 10% of cells were stained, the tumor was considered AKT-positive.

BrdU Incorporation and Indirect Immunofluorescence

For the 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdU) incorporation assay cells were grown to subconfluence on coverslips, incubated with 10 μ mol/L BrdU for 2 hours, fixed in 3% paraformaldehyde, and permeabilized with 0.2% Triton X-100. We used Texas Red-conjugated secondary antibodies to reveal BrdU-positive cells, and fluorescein isothiocyanate-conjugated secondary antibodies to reveal p27^{kip1}-positive cells. Cell nuclei were identified by Hoechst staining. Fluorescence was visualized with a Zeiss 140 epifluorescent microscope equipped with filters that discriminated between Texas Red and fluorescein. All assays were performed three times in duplicate.

Protein Extraction, Western Blots, and Antibodies

The antibodies used in this study were: anti-p27^{kip1} (Transduction Laboratories), anti-AKT and anti-phospho AKT (Ser473) (New England Biolabs, Lake Placid, NY), anti-phosphothreonine 157 (anti-P-T157),^{12–14} anti-phosphothreonine 198 (anti-P-T198),⁴³ anti-phosphothreonine 187 (anti-P-T187), and anti-phosphoserine 10 (anti-P-S10) (Zymed Laboratories Inc., San Francisco, CA). Total proteins were prepared as described elsewhere.¹⁰ Nuclear or cytoplasmic proteins were extracted by lysing cells in ice-cold hypotonic buffer (0.2% Nonidet P-40, 10 mmol/L Hepes, pH 7.9, 1 mmol/L ethylenediaminetetraacetic acid, 60 mmol/L KCl). Nuclei were separated through a 30% sucrose cushion and lysed in hypertonic buffer (250 mmol/L Tris-HCl, pH 7.8, 60 mmol/L HCl). The immunoblotting procedure we used is described elsewhere.¹⁰

Flow Cytometry

Cell cycle distribution was analyzed by flow cytometry as described previously.⁴⁴ In brief, cells were harvested in phosphate-buffered saline (PBS) containing 2 mmol/L ethylenediaminetetraacetic acid, washed once with PBS, and fixed for 30 minutes in cold ethanol (70%). Fixed cells were washed once in PBS and permeabilized with 0.2% Tween 20 and 1 mg/ml RNase A in PBS for 30 minutes. They were then washed once in PBS and stained with 50 μ g/ml of propidium iodide (Roche, Basel, Switzerland). Stained cells were analyzed with a fluorescence-activated cell sorter (FACS) Calibur (Becton-Dickinson, Franklin Lakes, NJ), and the data were analyzed using a mod-fit cell cycle analysis program.

Semiquantitative Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR)

Total cellular RNA was isolated from cultured cell lines as described previously.⁴⁵ For RT-PCR analysis, RNA extracted from thyroid cancer cell lines was digested with RNase-free DNase RQ1 (Promega). mRNA was reverse-transcribed using random hexamers as primers and Mo-MLV RT at 37°C for 10 minutes according to the Perkin-Elmer user manual. The PCRs were performed in the same tube using p27^{kip1}- or actin-specific primers with 1.25 U *Taq* polymerase. Reactions were performed for 20 cycles as follows: 1 minute at 94°C for denaturation, 2 minutes at 55°C for annealing, and 2 minutes at 72°C for extension. Amplified DNA fragments were fractionated on a 2% polyacrylamide gel and hybridized with a ³²P-labeled human p27^{kip1} probe. p27^{kip1} primers were chosen to amplify a full-length transcript resulting in a DNA fragment of 595 bp (forward: 5'-ATGTCAAACGTGCGAGTGTCTAAC-3'; reverse, 5'-ACGTTTGACGTCTTCTGAGGCCAG-3'). Human actin primers were chosen to amplify a cDNA fragment of 220 bp (forward 2329 to 2345, 5'-ACTTCGAGCAAGAGATG-3'; reverse 2611 to 2630, 5'-GCGGATGTCCACGGTCACACT-3'). All cDNA probes

were radiolabeled with a random prime synthesis kit (Multi-Prime; Amersham Biosciences). Hybridization reactions were performed at 42°C in 50% formamide, 5% Denhardt's, 5 $\times$ SSPE 0.2% sodium dodecyl sulfate (SDS), and 100 μ g/ml of denatured sonicated salmon sperm DNA, with 2 $\times$ 10⁶ cpm/ml of hybridization solution. Filters were washed at 60°C twice in 2 $\times$ standard saline citrate, 0.2% SDS, twice for 30 minutes and subsequently, for the stringent washes, twice for 30 minutes each in 0.2 $\times$ standard saline citrate, 0.1% SDS.

Transfections

AKT constructs,⁴⁶ the EGFP-PTEN construct,³³ and the wild-type p27^{kip1} construct⁴⁴ are described elsewhere. The p27^{kip1}-T187A, p27^{kip1}-T157A, p27^{kip1}-T198A, p27^{kip1}-S10A, and p27^{kip1}-T157A/T198A mutants were generated by site-specific mutagenesis (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. Cells were transfected with Eugene 6 (Roche). The average transfection efficiency into NPA cells was ~30 to 40%. Phosphorothioate anti-sense oligodeoxynucleotides were: p27-AS, 5'-TGTCTCTCGCACGTTTGACAT-3'; p27-MS, 5'-GGTCTTCCTAGTGTACTCATC-3'. Oligonucleotides were used at a concentration of 200 nmol/L and were delivered by the oligofectamine reagent (Invitrogen SRL, Milan, Italy).

In Vitro Degradation of p27^{kip1} Protein

In vitro degradation of p27^{kip1} protein was performed essentially as described previously.⁴⁷ Briefly, subconfluent or confluent TPC-1, NPA, WRO, FRO, and FB1 cells were grown, collected, and immediately frozen at -80°C. Protein extracts were prepared as described previously¹⁷ and incubated (100 μ g) with 1 μ g of recombinant His-tagged p27^{kip1} protein. After the indicated times, reactions were stopped by adding 1 vol of Laemmli buffer and loaded onto 12.5% polyacrylamide gel. p27^{kip1} protein was visualized with an anti-p27^{kip1} monoclonal antibody and then quantified by scanning of films.

Statistical Analysis

We used the χ^2 test with Yates correction or the two-tailed Fisher's exact test as appropriate. Data were analyzed with standard statistical software (SPSS version 9; SPSS, Chicago, IL). A probability value <0.05 was considered statistically significant.

Results

The PTEN-PI3K-AKT Pathway Regulates the Growth of Thyroid Cancer Cells through Control of p27^{kip1} Activity

To investigate the role of the PTEN-PI3K/AKT pathway in the regulation of thyroid cancer cell proliferation we examined the effects exerted by various PI3K inhibitors on

Figure 1. PTEN regulates the growth of thyroid cancer cells. **A:** Expression of EGFP-PTEN in NPA cells. Duplicate dishes of NPA cells (1 to 3×10^6 cells/10-cm dish) were transfected with $10 \mu\text{g}$ of pEGFP (lane 1) or pEGFP-PTEN (lane 2) to track transfected and untransfected cells. Forty-eight hours after transfection, cells were either lysed and subjected to immunoblotting using anti-PTEN antibodies or harvested and processed for flow cytometry. **B:** Left column, cells transfected with pEGFP control vector; middle column, cells transfected with pEGFP-PTEN, the cell cycle profile is that of EGFP-PTEN-negative cells; right column, cells transfected with EGFP-PTEN, the cell cycle profile is that of EGFP-PTEN-positive cells.

cell cycle progression and on $p27^{\text{kip1}}$ expression and localization in thyroid cancer cells. First, we transfected NPA cells with control empty pEGFP vector or fused pEGFP-PTEN ($10 \mu\text{g}$) to track transfected and untransfected cells. Cells were harvested 48 hours after transfection and the expression of transfected plasmids was determined by Western blotting using anti-PTEN antibody (Figure 1A). Transfected cells were also processed for flow cytometry. Green fluorescence was used to distinguish transfected from nontransfected cells. Figure 1B shows the cell cycle profile of cells transfected with pEGFP and control pcDNA3 vector (EGFP+), of EGFP-negative EGFP-PTEN-transfected cells (EGFP-PTEN-), and of EGFP-positive pEGFP-PTEN-transfected cells (EGFP-PTEN+). Overexpression of EGFP-PTEN resulted in accumulation of NPA cells in the G_1 phase of the cell cycle. In parallel, immunofluorescence experiments with EGFP- or EGFP-PTEN-transfected NPA cells showed that PTEN induced accumulation of nuclear $p27^{\text{kip1}}$ (see Figure 3).

To verify the results obtained with PTEN we determined the effects exerted by pharmacological inhibitors of PI3K (LY294002 and wortmannin) on five cell lines established from human thyroid carcinomas: two from PTCs (TPC-1 and NPA), one from an FTC (WRO), and two from ATCs (FRO and FB1). Exposure to $20 \mu\text{mol/L}$ of LY294002 or $0.2 \mu\text{mol/L}$ of wortmannin for 24 hours blocked proliferation and caused cells to accumulate in the G_1 phase of

Table 1. Flow Cytometry Analysis of Thyroid Cell Lines

Cells	Control	LY294002	Wortmannin
TPC-1			
G_0/G_1	66.5	94.7	90.1
S	24.3	1.5	7
G_2/M	9.0	3.8	2.9
NPA			
G_0/G_1	52.9	81.8	78.8
S	31.9	15.1	14.2
G_2/M	14.5	2.2	7
WRO			
G_0/G_1	73	91	93
S	21	5	4
G_2/M	6	4	3
FRO			
G_0/G_1	69	85.3	84
S	24.6	7.6	11.8
G_2/M	5.8	7.1	4.2
FB-1			
G_0/G_1	51	67.2	74.2
S	23.4	5.1	10.1
G_2/M	24.8	27	15.8

the cell cycle, as assessed by flow cytometry analysis. As shown in Table 1, LY294002 and wortmannin inhibited proliferation to a similar extent.

We investigated whether $p27^{\text{kip1}}$ was a downstream effector of the PI3K/AKT pathway in thyroid cancer cells by determining the effect of LY294002 on the same five cell lines (NPA, TPC-1, WRO, FRO, and FB1) in the presence of anti-sense oligodeoxynucleotides that blocked $p27^{\text{kip1}}$ synthesis. Cells were plated onto glass coverslips, oligofected with $p27^{\text{kip1}}$ anti-sense or control oligonucleotides (200 nmol/L), treated with LY294002 for 24 hours, incubated with $10 \mu\text{mol/L}$ BrdU for 2 hours, and processed for indirect immunofluorescence. A typical experiment is shown in Figure 2A. Inhibition of PI3K potently suppressed proliferation of thyroid cancer cells (60 to 90% of inhibition). Anti-sense oligonucleotides to $p27^{\text{kip1}}$ abolished most of the proliferative inhibition exerted by LY294002. Control oligonucleotides with scrambled sequences had no effect on the fraction of thyroid cancer cells that incorporated BrdU (not shown). These results indicate that $p27^{\text{kip1}}$ is a downstream effector of the PI3K/AKT pathway. The effects of anti-sense oligonucleotides on $p27^{\text{kip1}}$ expression are shown in Figure 2B.

The PI3K Pathway Regulates $p27^{\text{kip1}}$ Expression by Controlling Skp2 Levels

Treatment of thyroid cancer cells with LY294002 suppressed AKT phosphorylation at S473, but did not affect the total levels of AKT protein and, at most, resulted in a moderate increase (approximately twofold on average) in the total levels of $p27^{\text{kip1}}$ in all cell lines analyzed (Figure 3A). We next investigated the mechanisms underlying the PI3K-dependent regulation of $p27^{\text{kip1}}$ expression in TPC-1, NPA, WRO, FRO, and FB1 cells. Because the results were similar in all cell lines, we report only the data obtained with FB1 cells (Figure 4). FB1 cells were treated with LY294002 for 24 hours, washed free of LY294002, and $p27^{\text{kip1}}$ levels were monitored by immunoblot at 6, 8,

Figure 2. p27^{kip1} is a key molecular target of the PI3K/AKT pathway in the regulation of growth of thyroid cancer cells. **A:** TPC-1, NPA, WRO, FRO, and FB-1 cells were plated onto coverslips and treated with LY294002 for 24 hours in the presence of either anti-sense oligonucleotides to p27^{kip1} or control oligonucleotides with scrambled sequences, and incubated for 2 hours with 10 μ mol/L BrdU. Cells were fixed and processed for indirect immunofluorescence. At least 500 cells were counted. **B:** Western blot analysis of p27^{kip1} expression in TPC-1, NPA, WRO, FRO, and FB-1 cells treated or not with anti-sense oligodeoxynucleotides to p27^{kip1}.

and 24 hours. AKT inhibition resulted by LY2949002 in a slight to moderate p27^{kip1} accumulation, which was reversed by removal of LY294002 (at 8 and 24 hours) (Figure 4A). Using RT-PCR analysis we next determined whether the PI3K/AKT pathway regulates p27^{kip1} expression at mRNA level. Treatment of thyroid cancer cells with 20 μ mol/L LY294002 resulted in only a slight increase in p27^{kip1} mRNA level (Figure 4B). This suggests that the PI3K/Akt pathway regulates p27^{kip1} expression mostly at the protein level. Accordingly, LY294002 did not induce any clear change in the phosphorylation status of the forkhead transcription factors FoxO3a and FoxO4 (Figure 4C), which have been implicated in the regulation of p27^{kip1} transcription in hematopoietic cells.

To evaluate whether the PI3K/AKT pathway regulates the stability of p27^{kip1} protein in thyroid cancer cells, we treated FB1 cells with dimethyl sulfoxide (DMSO) or 20 μ mol/L LY294002 for 24 hours ($t = 0$). We then blocked translation of p27^{kip1} mRNA with 10 μ g/ml cycloheximide at 2, 4, and 8 hours. Cell lysates were prepared and equivalent amounts of proteins were loaded on SDS-polyacrylamide gel electrophoresis (PAGE) and probed with an anti-p27^{kip1} antibody. As shown in Figure 4D, p27^{kip1} protein disappeared faster in DMSO-treated cells than in LY294002-treated FB1 cells. This indicates that p27^{kip1} degrades faster in cells that have high PI3K ac-

Figure 3. PI3K/AKT controls p27^{kip1} protein levels in primary thyroid cancers. **A:** Indirect immunofluorescence analysis of p27^{kip1} localization in NPA cells transfected with EGFP (left column) or EGFP-PTEN (right column). EGFP- or EGFP-PTEN-transfected cells were identified by green fluorescence; endogenous p27^{kip1} was identified by Texas Red-conjugated antibody. **B:** Western blot analysis of PI3K-dependent p27^{kip1} expression in TPC-1, NPA, WRO, FRO, and FB-1 cells. Cells were treated with DMSO (lane C) or LY294002 (lane LY) for 24 hours, lysed, and subjected to immunoblot analysis for S473 P-AKT, total AKT, β -tubulin, or p27^{kip1}. The levels of p27^{kip1} protein are reported below each column as optical density units with p27^{kip1} levels in untreated cells being, arbitrarily, 1.

tivity. In fact, densitometry showed that the half-life of p27^{kip1} was $\sim$ 2 hours in cells with very active PI3K and longer than 8 hours in cells with low PI3K activity. Accordingly, proteasome extracts prepared from DMSO-treated cells and incubated at 37°C with 1 μ g of recombinant p27^{kip1} degraded recombinant p27^{kip1} faster than extracts from cells treated with 20 μ mol/L LY294002 (Figure 4E). Finally, immunoblot analysis demonstrated that inhibition of PI3K with LY294002 decreased Skp2 expression in thyroid cancer cell lines (Figure 4F). Taken together, these results suggest that in thyroid cancer cells the PI3K/AKT pathway regulates the rate of p27^{kip1} degradation by the 26S proteasome through the control of Skp2 expression.

AKT Phosphorylates p27^{kip1} at T157 and T198 in Thyroid Cancer Cells

The PI3K/AKT pathway also regulates the subcellular localization of p27^{kip1}.⁷ Indeed, the most striking conse-

Figure 4. PI3K/AKT controls $p27^{kip1}$ expression by regulating Skp2 protein expression. **A: Top**, immunoblot analysis of PI3K-dependent $p27^{kip1}$ expression in FB1 cells. Cells were treated with solvent alone (lane 1) or with 20 $\mu\text{mol/L}$ LY294002 for 24 hours (lane 2), washed free of LY294002, and cultured for 6, 8, and 24 hours in the absence of LY294002. **B**, Total cellular RNA was prepared from FB1 cells treated with DMSO or LY294002 (20 $\mu\text{mol/L}$) for 8 or 24 hours, as described in Materials and Methods. RNA (0.5 μg each) was reverse-transcribed and subjected to semiquantitative RT-PCR, fractionated onto 2% agarose gel, blotted onto nylon membranes, and revealed using ^{32}P -labeled $p27^{kip1}$ cDNA probe. **C**, Western blot analysis of PI3K-dependent expression and phosphorylation of FoxO3a and FoxO4 transcription factors in FB1 cells treated with DMSO or LY294002 (20 $\mu\text{mol/L}$) for 24 hours. **D**, FB1 cells were treated with DMSO or LY294002 (20 $\mu\text{mol/L}$) for 24 hours. To determine the half-life of $p27^{kip1}$ protein, the translation of $p27^{kip1}$ was blocked with 10 $\mu\text{g/ml}$ cycloheximide and cell lysates were prepared at 0, 2, 4, and 8 hours, loaded onto SDS-PAGE, and probed with an anti- $p27^{kip1}$ antibody. **E**, Rate of $p27^{kip1}$ degradation in extracts from FB1 cells treated with DMSO or LY294002 (20 $\mu\text{mol/L}$) for 24 hours. One μg of recombinant $p27^{kip1}$ was incubated at 37°C with 100 μg of proteasome extracts supplemented with 1 mmol/L ATP, 25 mmol/L phosphocreatine and 10 $\mu\text{g/ml}$ creatine kinase at 30°C for 0, 8, 16, and 24 hours, and the subsequent immunoblot analysis revealed the amount of intact $p27^{kip1}$ protein in different conditions. **F**, Skp2 expression in control (C) or LY294002 (LY)-treated FB1 cells.

quence of LY294002 in thyroid cancer cells was a change in $p27^{kip1}$ subcellular localization (Figure 5). To compare the amounts of cytoplasmic and nuclear $p27^{kip1}$ in control and LY-treated cells, we expressed them as cytoplasmic-to-nuclear protein ratio. In proliferating DMSO-treated cells, cytoplasmic $p27^{kip1}$ ranged from a ratio of 1:1 in TPC-1 cells to less than 10:1 in NPA and FRO cells. On PI3K block, the fraction of nuclear $p27^{kip1}$ markedly increased (the ratio ranged from 1:2 in TPC-1 cells to 1:100 in FB-1 cells). Nuclear accumulation of $p27^{kip1}$ subsequent to PI3K block was paralleled by an increase of the $p27^{kip1}$ fraction complexed with nuclear CDK2 and inhibition of CDK2 activity (not shown). This indicates that $p27^{kip1}$ accumulation inhibited cell proliferation.

Figure 5. PI3K/AKT controls $p27^{kip1}$ localization in thyroid cancer cells. Immunoblot analysis of PI3K-dependent $p27^{kip1}$ subcellular localization in thyroid cancer cells. TPC-1, NPA, FRO, and FB-1 cells were treated with DMSO or LY294002 (20 $\mu\text{mol/L}$) for 24 hours, lysed and fractionated into cytoplasmic (cyt) and nuclear (nuc) fractions to determine $p27^{kip1}$ localization. Anti-SP1 and β -tubulin antibodies were used as controls.

AKT, a serine/threonine kinase downstream from PI3K, mediates the relocalization of $p27^{kip1}$ in breast cancer cells.^{12–14} Therefore, we investigated whether AKT mediated the effects exerted by the PI3K pathway on the subcellular localization of $p27^{kip1}$ in thyroid cancer cells. NPA cells were stably transfected with control vector or the constitutively active myristylated AKT allele, myrAKT. After transfection, NPA-AKT cells were selected in G418 and two clones (clones 6 and 10) were expanded for biological studies. NPA-AKT clones 6 and 10 gave essentially similar results; thus we will refer to them as “NPA-AKT” cells. NPA and NPA-AKT cells were seeded onto coverslips, grown for 2 days, and treated with DMSO or LY294002 (20 $\mu\text{mol/L}$) for 24 hours. Then cells were incubated with 10 $\mu\text{mol/L}$ BrdU for 2 hours and processed for indirect immunofluorescence. As shown in Figure 6A, BrdU incorporation was greater in NPA-AKT cells than in parental NPA and NPA-CMV-neo cells, especially in the presence of LY294002 (23.5% of NPA-AKT cells incorporated BrdU versus 14% of NPA cells). Treatment with LY294002 of NPA cells induced marked accumulation of $p27^{kip1}$ in cell nuclei (Figure 5B, left), whereas stable expression of activated AKT in NPA cells (NPA-AKT cells) reduced LY294002-dependent $p27^{kip1}$ nuclear accumulation (Figure 6B, right).

AKT-dependent localization of $p27^{kip1}$ is regulated by the phosphorylation of one of three key residues, S10, T157, and 198.⁷ We used phospho-specific antibodies to investigate whether the PI3K/AKT pathway regulates $p27^{kip1}$ localization in thyroid cancer cells through AKT-dependent phosphorylation of T157, T198, or S10. Using the anti-P-T157 and anti-P-T198 antibodies we found

Figure 6. Activated AKT prevents LY294002-dependent growth arrest of thyroid cancer cells by modulating p27^{kip1} localization. **A:** BrdU incorporation of NPA and NPA-AKT cells treated with DMSO or LY294002 (20 μ mol/L) for 24 hours, incubated with 10 μ mol/L BrdU for 2 hours, and processed for indirect immunofluorescence. **B:** Immunoblot analysis of PI3K-dependent p27^{kip1} subcellular localization in thyroid cancer cells. NPA and NPA-AKT cells were treated with DMSO or LY294002 (20 μ mol/L) for 24 hours, lysed, and fractionated into cytoplasmic (cyt) and nuclear (nuc) fractions to determine p27^{kip1} localization. Anti-SP1 and β -tubulin antibodies were used as controls.

p27^{kip1} phosphorylated at both these sites in proliferating NPA cells (Figure 7A, lane 1); exposure for 24 hours to LY294002 inhibited AKT and reduced phosphorylation of T157 and T198 (Figure 7A, lane 2). Conversely, treatment with a PI3K inhibitor (LY294002) did not affect the phosphorylation status of S10. It has been suggested that S10 is phosphorylated by AKT⁴⁸ and it has been shown to be critical for p27^{kip1} cytoplasmic export.^{23,24} The finding that p27^{kip1} phosphorylation of T157 and T198 was enhanced in NPA-AKT cells versus NPA cells (Figure 7A; compare lanes 3, 4, and 1, 2) is the formal demonstration that in thyroid cancer cells these sites are phosphorylated in an AKT-dependent manner. As in the case of breast cancer,^{14,43} phosphorylation of endogenous p27^{kip1} at T157 and T198 in NPA cells was restricted almost exclusively to the cytoplasmic compartment (Figure 7B).

Phosphorylation of p27^{kip1} at T157 and T198 Is Required for AKT Regulation of p27^{kip1} Localization in Thyroid Cancer Cells

We next investigated whether the various AKT-phosphorylated residues are crucial for the regulation of the sub-

Figure 7. The PI3K/AKT pathway controls p27^{kip1} phosphorylation at T157 and T198 in thyroid cancer cells. **A:** NPA and NPA-AKT cells were treated with DMSO or LY294002 (20 μ mol/L) for 24 hours and lysed. Total proteins (1 μ g) were immunoprecipitated with a polyclonal anti-p27^{kip1} antibody, separated onto SDS-PAGE, and immunoblotted with S10, T157, and T198 phospho-specific antibodies, as indicated. Immunoprecipitates were normalized by immunoblot with a monoclonal anti-p27^{kip1} antibody. Antibody to P-Akt S473 was used in immunoblots on lysates (40 μ g) as control of the Akt activation status. **B:** NPA-AKT cells were treated with DMSO or LY294002 (20 μ mol/L) for 24 hours and lysed. Cytoplasmic or nuclear proteins were immunoprecipitated with a polyclonal anti-p27^{kip1} antibody, separated onto SDS-PAGE, and immunoblotted with T157 or T198 phospho-specific antibodies, as indicated. Immunoprecipitates were normalized by immunoblot with monoclonal anti-p27^{kip1} antibody. Anti-SP1 and β -tubulin antibodies were used in immunoblots on lysates (40 μ g) derived from cytoplasmic or nuclear proteins as controls of fractionation.

cellular distribution of p27^{kip1}. To address this issue, we transfected HA-tagged wild-type and mutant p27^{kip1} in thyroid cancer cells (NPA) and evaluated the effects induced by pharmacological modulation of the PI3K pathway on p27^{kip1} localization. NPA cells were plated onto coverslips and transfected with wild-type HA-p27^{kip1}, or with mutant HA-p27^{kip1}-T157A, HA-p27^{kip1}-T198A, HA-p27^{kip1}-T157A-T198A, or HA-p27^{kip1}-S10A. Twenty-four hours later the cells were treated with DMSO or LY294002 (20 μ mol/L) to suppress endogenous PI3K activity for an additional 24 hours. p27^{kip1} localization was determined by indirect immunofluorescence. The average results of three independent experiments are shown in Figure 8A; a representative experiment is shown in Figure 8B.

In NPA cells, wild-type p27^{kip1} localized almost entirely to the cytoplasm, thereby mimicking endogenous p27^{kip1} protein (see Figure 3) (~83% of cells showed cytoplasmic staining). However, in LY294002-treated NPA cells, wild-type p27^{kip1} was mainly nuclear (<20% of cells showed cytoplasmic staining). Of the mutant constructs, HA-p27^{kip1}-T157A showed reduced cytoplasmic localization in the absence of LY294002 (33% of cells showed cytoplasmic staining) but the mutant was relocalized to the nucleus in the presence of LY294002 (~20% of cells

Figure 8. Phosphorylation of p27^{kip1} at T157 and T198 in thyroid cancer cells is critical for regulation of p27^{kip1} localization by the PI3K/AKT pathway. PCDNA3 plasmids (1 μ g) encoding HA-tagged wild-type or mutant T157A, T198A, S10A p27^{kip1} were transfected into NPA cells and treated with DMSO or LY294002. Forty-eight hours later cells were fixed, permeabilized, and processed for indirect immunofluorescence. Monoclonal anti-p27^{kip1} antibodies (K25020, dilution 1:10; Transduction Laboratories) were used to reveal the localization of the p27^{kip1} mutants. Hoechst identified cell nuclei. The graph represents the median of three experiments. **Red bars**, percentage of cells with cytoplasmic p27^{kip1}; **blue bars**, percentage of cells with nuclear p27^{kip1}. **B:** Representative immunofluorescence with monoclonal anti-p27^{kip1} antibody (K25020, dilution 1:10; Transduction Laboratories) revealed with fluorescein isothiocyanate-conjugated secondary antibodies. Nuclei were identified by Hoechst staining. Original magnifications, $\times 100$.

showed cytoplasmic staining). Similarly, HA-p27^{kip1}-T198A showed reduced cytoplasmic localization in the absence of LY294002 (only 43% of cells showed cytoplasmic staining) and was relocalized to the nucleus in the presence of LY294002 (~18% of cells showed cytoplasmic staining). The p27^{kip1}-T157A-T198A double mutant showed almost complete nuclear localization (~17% and 11% of cells showed cytoplasmic staining in the absence and presence of LY294002, respectively). As expected from the phosphorylation data of Figure 7, the subcellular localization of the S10A mutant did not differ significantly from that of the wild-type HA-p27^{kip1}, which is further confirmation that S10 phosphorylation is not involved in AKT-dependent p27^{kip1} relocalization. These data demonstrate that T157 and T198, but not S10, are involved in PI3K-dependent cytosolic relocalization of p27^{kip1}.

Figure 9. AKT activation in thyroid tumors **A:** Sections from thyroid cancers were subjected to immunohistochemistry for S473 P-AKT. **Left:** S473 P-AKT-negative papillary carcinoma. **Right:** S473 P-AKT-positive papillary carcinoma. **Inset:** Western blot analysis of S473 P-AKT in the same tumors. **B:** AKT activation in thyroid carcinomas.

Activation of the PI3K-AKT Pathway Is Associated with a Cytosolic Location of p27^{kip1} in Thyroid Cancer

The ability of AKT to modulate the expression and cellular localization of p27^{kip1} in established cell lines suggested that the presence of active AKT in thyroid tumors would correlate with the expression of cytosolic p27^{kip1}. To address this issue, we determined p27^{kip1} expression and localization in 100 specimens of thyroid carcinomas, and evaluated whether the data correlated with the activity of the PI3K/AKT pathway. We determined the status of the latter by measuring the extent of AKT phosphorylation in primary thyroid carcinomas (10 FAs, 62 PTCs, 23 FTCs, and 5 ATCs) by immunoblot experiments with the phospho-specific anti-S473 AKT antibody.

AKT activity was confirmed at single cell level by immunostaining of all but one sample. The results of immunostaining coincided with immunoblot results. P-AKT was detected in both the nucleus and cytoplasm of tumor cells but was absent from stromal cells (Figure 9A). Malignant tumors presented cytoplasmic P-AKT staining

Table 2. Association between AKT Activation and p27^{kip1} Expression in Thyroid Tumors

	AKT-negative tumors* (n = 57)	AKT-positive tumors* (n = 43)
Low p27 ^{kip1} expression, n (%)	31 (54.4)	20 (46.5)
High p27 ^{kip1} expression, n (%)	26 (45.6)	23 (53.5)

AKT activation was measured by immunoblot with anti-S473 phospho-antibody. p27^{kip1} expression was measured by immunoblot.
 *Statistical analysis, performed with the χ^2 test with Yates correction, yielded a *P* value >0.05.

more often than benign tumors: AKT was activated in 1 of 10 FAs, 27 of 62 PTCs, 12 of 23 FTCs, and 4 of 5 ATCs (Figure 9B). The difference in AKT activation between adenomas (*n* = 10) and carcinomas (*n* = 90) was significant (*P* < 0.05; two-tailed Fisher's exact test). As shown in Figure 8B, the difference in the frequency of AKT activation in differentiated (39 of 85) versus anaplastic carcinomas (four of five) was not significant (*P* > 0.1; two-tailed Fisher's exact test).

We next evaluated tumors with low pAKT activity (57 of 100) and those with high pAKT activity (43 of 100), and found no correlation between AKT activation status and p27^{kip1} protein levels: p27^{kip1} expression was similarly low in the two groups (Table 2). To investigate whether there was a correlation between activated AKT and p27^{kip1} localization, we examined 33 primary thyroid tumors (7 FAs, 18 conventional PTCs, 7 FTCs, and 1 ATC) using immunohistochemistry and the anti-p27^{kip1} antibody (Table 3). Benign tumors (6 of 7 adenomas) had nuclear p27^{kip1} staining, and malignant tumors (13 of 18 PTCs, 4 of 7 FTCs) had cytoplasmic p27^{kip1} staining. Furthermore, P-AKT levels clearly correlated with p27^{kip1} localization: p27^{kip1} was exclusively nuclear in 14 of 17 P-AKT-negative specimens (82%), whereas p27^{kip1} was exclusively cytosolic in 16 of 18 (93%) of tumors with the greatest P-AKT staining (*P* < 0.002; χ^2 test with Yates correction) (Figure 10A). A representative experiment is shown in Figure 10B. The subcellular localization of p27^{kip1} was confirmed by immunoblot on cytoplasmic- and nuclear-enriched proteins from eight tumors with essentially nuclear p27^{kip1} staining and eight tumors with essentially cytoplasmic p27^{kip1} staining (Figure 10C).

Subsequently, we assessed T157 and T198 phosphorylation of p27^{kip1} using anti-P-T157 and anti-P-T198 immunoblots of p27^{kip1} immunoprecipitates (Table 4). Phosphorylation of p27^{kip1} at T157 and T198 occurred in six of seven tumors (three of three FTCs, two of three PTCs, and one of one ATC) (85%) with activated AKT, but in only two of nine tumors (one of three FTCs and one of six PTCs) (25%) with unphosphorylated AKT (Figure 11A). Figure 11B shows a Western blot with anti-phospho-T157, T198, and total p27^{kip1} antibodies. The correlation between AKT activation and T157/T198 phosphorylation of p27^{kip1} was significant (*P* < 0.05; two-tailed

Fisher's exact test). All tumors that were positive for phosphorylated p27^{kip1} had p27^{kip1} in the cytoplasmic compartment, whereas the tumors negative to the anti-phospho-antibodies had nuclear staining.

Discussion

We have used five representative cell lines derived from PTCs, FTCs, and ATCs, and 100 human thyroid tumors to investigate the role of the PI3K/AKT pathway in the development of thyroid cancer. This pathway has been implicated in thyroid tumorigenesis in the clinical setting. In fact, there is a high frequency of thyroid carcinomas in patients affected by Cowden's disease, an autosomal-dominant multiorgan syndrome characterized by benign and malignant thyroid tumors, in which AKT is activated by germline loss of the negative regulator PTEN.⁴⁹ AKT is activated also in sporadic thyroid cancer either through loss of PTEN^{33,36} or increased mRNA and/or protein expression.^{37,38}

Here we provide compelling evidence that the PI3K/AKT pathway and its downstream effector p27^{kip1} play major roles in the process of thyroid carcinogenesis, in particular in thyroid cancer cell proliferation. This coincides with reports that sporadic thyroid tumors carry PTEN mutations and/or deletions and that the PI3K/AKT pathway is required for growth of thyrocytes in response to insulin, IGF-1, and serum.^{50–54} The novel finding of our study is that increased signaling through the PI3K/AKT pathway causes cytoplasmic mislocalization of p27^{kip1} in thyroid cancer cells. In fact, in these cells, phosphorylation and localization of p27^{kip1} is regulated in a PI3K- and AKT-dependent manner. Activated AKT significantly correlated with p27^{kip1} cytoplasmic mislocalization, whereas p27^{kip1} was exclusively nuclear in tumors with inactive AKT.

The mechanism regulating p27^{kip1} localization in human cancer is still primarily obscure. Nuclear import of p27^{kip1} requires the presence of a nuclear localization signal at the C-terminus of the protein.^{54,55} Cytoplasmic redistribution of p27^{kip1} requires the phosphorylation of at least one of three residues (S10, T157, and T198).^{12–14} The human kinase interacting stathmin (hKIS) is required

Table 3. Association between AKT Activation and p27^{kip1} Localization in Thyroid Tumors

	AKT-negative tumors* (n = 17)	AKT-positive tumors* (n = 16)
Nuclear p27 ^{kip1} localization, n (%)	14 (82.4)	1 (6.3)
Cytoplasmic p27 ^{kip1} expression, n (%)	3 (17.6)	15 (93.7)

AKT activation was measured by immunoblot with anti-S473 phospho-antibody. p27^{kip1} immunostaining was performed with monoclonal anti-p27^{kip1} antibody.
 *Statistical analysis, performed using the two-tailed Fisher's exact test, yielded a *P* value <0.002.

Figure 10. Cytoplasmic mislocalization of p27^{kip1} in primary thyroid cancer is associated with AKT activation. **A:** Correlation between AKT activation and nuclear (blue bars) or cytoplasmic (red bars) p27^{kip1} subcellular localization. Low: tumors with low AKT S473 phosphorylation. High: tumors with high AKT S473 phosphorylation. **B:** Sections from 33 thyroid cancers were subjected to immunohistochemistry for S473 P-AKT and p27^{kip1}. **Top row:** S473 P-AKT-negative papillary carcinoma (left) with p27^{kip1} expression only in cell nuclei (right). **Bottom row:** S473 P-AKT-positive papillary carcinoma (left) with p27^{kip1} expression in the cytoplasm of tumor cells (right). **C:** Western blot analysis of p27^{kip1} localization on nuclear- and cytoplasmic-enriched protein extracts from the same samples as in **B**.

for S10 phosphorylation,^{23,24} whereas AKT is required for phosphorylation of T157 and T198.^{12–14} By phosphorylating p27^{kip1} on T157 and T198, AKT induces binding of p27^{kip1} to 14.3.3,^{48,55} prevents binding to importin- α ,¹² impairs nuclear import,^{12,55} and overcomes p27^{kip1}-induced growth arrest.^{12–14}

We used specific phospho-antibodies and transfection experiments to determine the role of site-specific phosphorylation in the PI3K/AKT-dependent regulation of p27^{kip1} localization in thyroid cancer cells. The inhibition of the PI3K/AKT pathway in thyroid cancer cells coordinately increased p27^{kip1} nuclear relocalization and reduced p27^{kip1} phosphorylation at T157 and T198,

Figure 11. Phosphorylation of p27^{kip1} at T157 or T198 in primary thyroid cancer is associated with AKT activation. **A:** Correlation between AKT activation and T157 (blue bars) or T198 (red bars) p27^{kip1} phosphorylation. Low: tumors with low AKT S473 phosphorylation. High: tumors with high AKT S473 phosphorylation. **B:** AKT S473 phosphorylation and phosphorylation of p27^{kip1} at T157 and T198 in primary thyroid cancers. For phosphorylation analysis, total proteins (2 mg) were immunoprecipitated with a polyclonal anti-p27 antibody, separated onto SDS-PAGE, and immunoblotted with T157 or T198 phospho-specific antibodies. Immunoprecipitates were normalized by immunoblot with a monoclonal anti-p27^{kip1} antibody. The status of P-AKT and AKT was determined by immunoblot.

whereas it did not affect p27^{kip1} phosphorylation at S10. The finding that both nuclear relocalization of p27^{kip1} and the reduction in T157 and T198 phosphorylation induced by LY294002 was prevented by overexpressing constitutively active AKT in NPA cells indicates that excessive AKT-dependent phosphorylation of p27^{kip1} at T157 and T198 is a major mechanism of cytoplasmic mislocalization of p27^{kip1} in thyroid cancer. In support of this con-

Table 4. Association between AKT Activation and p27^{kip1} Phosphorylation in Thyroid Tumors

	AKT-negative tumors* (n = 9)	AKT-positive tumors* (n = 7)
Phosphorylated p27 ^{kip1} , n (%)	2 (22.2)	6 (85.7)
Unphosphorylated p27 ^{kip1} , n (%)	7 (77.8)	1 (14.3)

AKT activation was measured by immunoblot with anti-S473 phospho-antibody. p27^{kip1} phosphorylation was measured by immunoblot with anti-T157 or T198 phospho-antibodies.

*Statistical analysis, performed using the two-tailed Fisher's exact test, yielded a *P* value < 0.05.

clusion, we found that AKT was activated in ~45% of thyroid carcinomas and that the presence of P-AKT in thyroid cancer was significantly associated with p27^{kip1} phosphorylation at T157 and T198 ($n = 16$, $P < 0.05$) and with its cytoplasmic localization ($n = 33$, $P < 0.002$). Conversely, in tumors with inactive AKT, T157, and T198 phosphorylation of p27^{kip1} was low and p27^{kip1} was found only in nuclei. This scenario is reminiscent of breast cancer, in which AKT activation has been reported to lead to p27^{kip1} phosphorylation at T157 and T198 and subsequent cytoplasmic relocation.^{12–14,43}

Our experiments with p27^{kip1} mutants provide further evidence that the T157A and T198A sites contribute to the cytoplasmic retention of p27^{kip1} in thyroid cancer cells. In fact, the cytoplasmic location of mutants T157A and T198A was reduced versus wild-type p27^{kip1} in NPA cells in the absence of LY294002, although LY294002 still increased the capacity for relocation to the nucleus. Only the double-mutant p27^{kip1}-T157A-T198A was located exclusively in the nuclear compartment irrespective of LY294002. This finding is in agreement with the recent observation that both T157 and T198 are required for binding to 14.3.3 proteins, the cytoplasmic anchors that keep p27^{kip1} in the cytoplasmic compartment.⁵⁵

What is the function of the nuclear-to-cytoplasmic mislocalization of p27^{kip1} in the development of thyroid tumors? It has been suggested that impaired import of p27^{kip1} into cell nuclei lowers the nuclear concentration of p27^{kip1} under a critical threshold thereby preventing p27^{kip1}-induced inhibition of cyclin E-Cdk2 activity.¹⁰ However, a broader analysis of the literature supports the idea that p27^{kip1} exerts some oncogenic cytoplasmic functions that foster carcinogenesis.⁵⁶ Indeed, in many thyroid tumors p27^{kip1} is not simply lost but is mislocalized. In analogy with the related Cdk inhibitor p21^{cip1},⁵⁷ cytoplasmic p27^{kip1} may suppress apoptosis or regulate migration, thus allowing cancer cells to dysregulate multiple cellular functions with one hit. Accordingly, the presence of cytoplasmic p27^{kip1} has recently been associated with increased migration in AKT-expressing thyroid cancer cells.³⁸

Activation of the PI3K/AKT pathway has been implicated in the regulation of p27^{kip1} expression in diverse cell lines.⁵⁸ AKT can inhibit p27^{kip1} gene expression by targeting the forkhead transcription factor FoxO4 (formerly "AFX") in fibroblasts and hematopoietic cells^{58–60} and by regulating p27^{kip1} protein stability.⁶¹ Our results demonstrate that in thyroid cancer cells, the PI3K pathway regulates p27^{kip1} proteolysis by controlling the expression of Skp2 ubiquitin ligase, although regulation of p27^{kip1} expression in these cells is apparently AKT-independent. In fact, AKT activation is not apparently associated with reduced p27^{kip1} expression in tumors and p27^{kip1} expression is not lower in NPA-AKT cells than in NPA cells (not shown). Therefore, other molecules downstream and/or parallel PI3K (ie, MAP kinase) may account for p27^{kip1} degradation in thyroid cancer cells.

The PI3K/AKT signaling pathway contributes to the malignant progression of human cancer.²⁵ In thyroid cancer, PI3K/AKT is activated in 40 to 50% of differentiated carcinomas (PTCs and FTCs) and in 80% of ATCs, and is

thus involved in all types of thyroid carcinomas. Ringel and colleagues³⁷ first found AKT activation, determined by immunoblot, in FTCs (but not in PTCs). They later reported activated AKT in most PTCs analyzed.³⁸ As mentioned above, PTCs, FTCs, and ATCs result from different genetic lesions,²⁷ which in turn lead to the differential activation of downstream signaling pathways (such as PI3K/AKT or MAP kinase) and, as a consequence, to different effects on p27^{kip1} expression and/or localization. Our results lead us to expect cytoplasmic sequestration of p27^{kip1} in tumors that present either PTEN deletion or AKT hyperactivity but not in tumors with activated BRAF. Conversely, it will be difficult to predict p27^{kip1} localization in tumors that harbor upstream genetic alterations, such as RET/PTC rearrangements or RAS mutations, which activate both the MAP kinase and PI3K/AKT cascades. Indeed, oncogenic RAS induces p27^{kip1} loss in human normal thyrocytes.⁶² Similarly, activated RET/PTC caused MAP kinase-dependent downregulation of p27^{kip1} expression in rat and human thyroid cells, and pharmacological inhibition of endogenous or transfected RET/PTC restored p27^{kip1} expression.⁶³

In conclusion, our study conducted with cultured cell lines and human thyroid tumors casts light on the intracellular pathways that impair the inhibitory function of p27^{kip1} in thyroid carcinogenesis.

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