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"A novel mechanism of inactivation of cycline-dependent kinase inhibitor $p27^{Kip1}$ in thyroid cancer: cytoplasmic delocalization induced by the protein kinase Akt"

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A novel mechanism of inactivation of cycline-dependent kinase inhibitor $p27^{kip1}$ in thyroid cancer: cytoplasmic delocalization induced by the protein kinase Akt.

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3. D. Vitagliano, F. Carlomagno, **ML Motti**, G Viglietto, YE Nikiforov, MN Nikiforova, JM Hershman, AJ Ryan, A Fusco, RM Melillo, and M Santoro. Regulation of p27kip1 protein level contributes to mitogenic effects of the RET/PTC kinase in thyroid carcinoma cells. *Cancer Research* (2004). June 1, 64(11): 3823-9.

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5. Di Vizio D, Cito L, Boccia A, Chieffi P, Insabato L, Pettinato G, **Motti ML**, Schepis F, D'Amico W, Fabiani F, Tavernise B, Venuta S, Fusco A, Viglietto G. Loss of the tumor suppressor gene PTEN marks the transition from intratubular germ cell neoplasias (ITGCN) to invasive germ cell tumors. Oncogene. 2005; 24(11): 1882-94.

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8. **ML Motti**, G Baldassare, A Celetti, F Merolla, D Califano, F Forzati, M Napolitano A Fusco and G Viglietto Dysfunction of E-cadherin/ -catenin system causes loss of $p27^{kip1}$-mediated contact inhibition in thyroid anaplastic carcinomas. Carcinogenesis 2005; 26(6): 1021-1034.

Abstract

The cyclin-dependent kinase inhibitor p27 is a putative tumor suppressor for human cancer. There is considerable evidence that the inactivation of the cyclin-dependent kinase inhibitor p27 is a fundamental step for the development of human malignancies. The inactivation of the tumor suppressor $p27^{kip1}$ in human cancer occurs either through loss of expression or phosphorylation-dependent cytoplasmic sequestration. The work done during my Doctorate thesis has contributed to a better understanding of the alterations of p27 function in human thyroid (and breast) cancer and to the identification of the different mechanisms that contribute to it.

The results presented here demonstrate that the serine/threonine kinase Akt regulates cell proliferation in thryoid cancer cells by directly phosphorylating p27. Threonine 157 (T157), which maps within the nuclear localization signal of p27, and threonine 198 (T198) represent the predicted Akt-phosphorylation sites. Akt-induced T157/T198 phosphorylation causes binding to 14.3.3 proteins and retention of p27 in the cytoplasm of cancer cells, precluding p27-induced G1 arrest. Conversely, the p27-T157A-T198A mutant does not bind 14.3.3, accumulates in cell nuclei and is insensitive to Akt-mediated mitogenic activity.

Experiments performed using five representative thyroid cancer cell lines have indicated that dysregulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway plays an important role in thyroid carcinogenesis and that p27 is a key target of the growth-promoting activity exerted by this pathway in thyroid cancer cells. Using specific inhibitors of PI3K (LY294002, wortmannin, PTEN), as well as dominant active Akt constructs (ca-AKT), we demonstrate that the PI3K/Akt-dependent control of thyroid cell proliferation occurs through regulation of p27 subcellular localization; that the localization of p27 is dependent on Akt-dependent phosphorylation of p27 at T157 and T198; that T157/T198-phosphorylated p27 resides exclusively in the cytoplasmic compartment; and, finally, that T157/T198-phosphorylated p27 accumulates in the cytoplasm of primary human thyroid cancer cells coincident with Akt activation.

Thus, we propose that the cytoplasmic relocalization of p27, which occurs upon Akt-mediated phosphorylation, is a novel mechanism whereby the growth inhibitory properties of p27 are functionally inactivated and the proliferation of thyroid cancer cells is sustained.

1. Background.

1. Cell Cycle

Recent advances in our understanding of the cell cycle machinery in the last years have demonstrated that disruption of normal cell cycle control is frequently observed in human cancer (Sherr et al.1995; Ortegaet al. 2002). Cell cycle progression is governed by a class of serine-threonine kinases, denoted cyclin-dependent kinases (Cdks) that are enzimatically activated by association with regulatory subunits called cyclins (Morgan et al. 1995). The ordered progression through G1 phase of the cell cycle is regulated by the sequential assembly and activation of three sets of cyclin-Cdk complexes: the D cyclins (D1, D2 and D3) and Cdk4 or Cdk6; cyclin E and Cdk2; cyclin A and Cdk2 (Ekholm et al. 2000). The cyclin D and E-dependent kinases contribute sequentially to the phosphorylation of the retinoblastoma gene susceptibility product (pRB), cancelling its ability to repress E2F transcription factors and activating genes required for S phase entry (Weinberg et al. 1995) (see Fig.1).

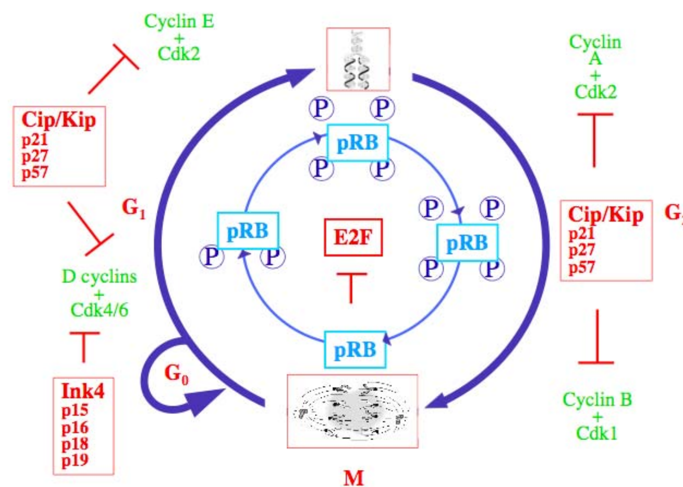


Fig.1: Cell cycle. Progression through cell cycle is regulated by the sequential assembly and activation of distinctive cyclin-Cdk complexes: D cyclins (D1, D2 and D3)/ Cdk4 or Cdk6; cyclin E/Cdk2; cyclin A/Cdk2; cyclin B/Cdk1. The cyclin D and E-dependent kinases contribute to the phosphorylation of pRB, abrogating its ability to repress E2F transcription factors. Two families of Cdk inhibitors have been defined: the Ink4 family (p15, p16, p18 and p19) and the Cip/Kip family (p21, p27 and p57).

The activity of these cyclin/Cdk complexes is regulated by positive and negative phosphorylation events executed by the Cdk Activating Kinase (CAK), by the Wee kinases and the CDC25 phosphatases, respectively (Morgan et al1995; Ekholm et al. 2000). Additional regulation of G1 Cdk

activity is affected by their association with inhibitory proteins called Cdk inhibitors (Ckis), that can either physically block activation or block substrate/ATP access (Sherr et al. 1995). So far, two families of Cki have been defined based on their structure and Cdk targets: the Ink4 family and the Cip/Kip family (Sherr et al. 1999). The inhibitors of Ink4 family (p15, p16, p18 and p19) bind to monomeric Cdk4 and Cdk6 but not to Cdk2, thereby precluding the association of these Cdks to cyclins D (Ortega et al. 2002). Conversely, the members of Cip/Kip family, that include p21, p27 and p57, all contain characteristic motifs at their N-terminal moieties, that enable them to bind both Cdk and cyclins (Sherr et al. 1999) (Fig.2).

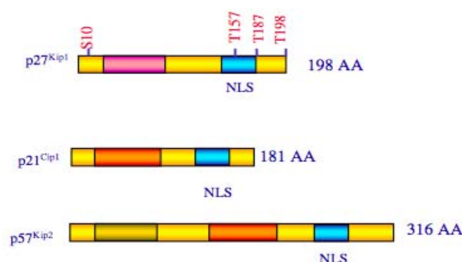


Fig 2: Structure of the members of the Cip/Kip family of inhibitors. NLS, Nuclear Localization Signal.

Although Cip/Kip inhibitors bind and inhibit complexes containing cyclin D, E and A and their Cdk partners, it is thought that the main target of Cip/Kip inhibitors is cyclin E-Cdk2 (Sherr et al.1999; Vidal et al. 2000).

2. Regulation of p27 activity

The Cip/Kip inhibitor p27 was initially discovered as an inhibitor of the cyclin E-Cdk2 complex (Polyak et al. 1994). Subsequently, p27 was shown to be a fundamental regulator of proliferation in most cell types, being deputed to maintain quiescence (Rivard et al. 1996). P27 acts primarily in G0 and early G1, where it is required for G1 arrest induced by growth factor deprivation, contact inhibition and loss of adhesion to extracellular matrix (Coats et al. 1996). As a general rule, p27 expression is highest in quiescent cells and

declines upon mitogenic stimulation. Most anti-mitogenic compounds and differentiating agents increment the intracellular levels of p27, while exposure of epithelial and lymphoid cells to mitogenic growth factors or cytokines causes loss or reduction of p27 (Ekholm et al. 2000; Sherr et al. 1999; Vidal et al. 2000).
The regulation of p27 activity is complex: it occurs through the control of its intracellular concentration, its distribution among different cyclin-Cdk complexes and its subcellular localization (Olashaw et al. 2002). Three different mechanisms have been implicated in regulating p27 expression: transcriptional regulation of p27 promoter (Dijkers et al. 2000; Servant et al. 2000), control of mRNA translation (Hengst et al. 1996; Millard et al. 1997) and regulation of protein degradation rate (Pagano et al. 1995). Proteolysis through the ubiquitin-proteasome pathway is thought to be the predominant mechanism whereby p27 abundance inside cycling cells is determined and occurs at the G1/S transition (Fig. 3).

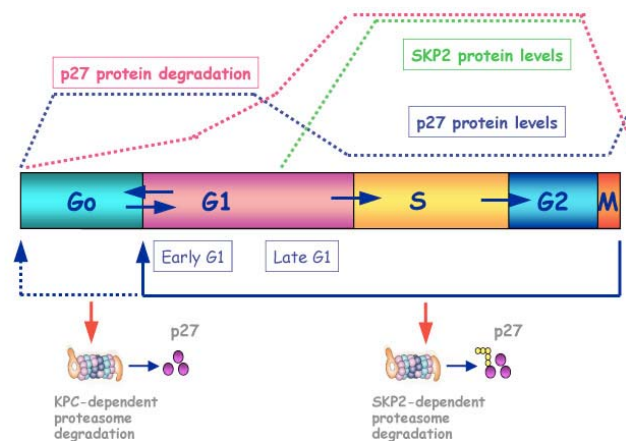


Fig 3: Regulation of p27 expression during cell cycle. In early and mid G1 p27 degradation is dependent on KPC whereas in late G1 and S phase its degradation is SKP2-dependent.

Degradation of p27 through the ubiquitin-proteasome pathway is a three-step process that requires: (i) phosphorylation of p27 at threonine 187 by cyclin E/Cdk2 (Sheaff et al. 1997; Nguyen et al. 1999); (ii) recognition of T187-phosphorylated p27 by the ubiquitin ligase SCF^{Skp2} (Montagnoli et al. 1999; Carrano et al. 1999); (iii) SCF^{Skp2}-dependent ubiquitination and degradation of T187-phosphorylated p27 (Pagano et al. 1995; Carrano et al. 1999) (Fig.4).

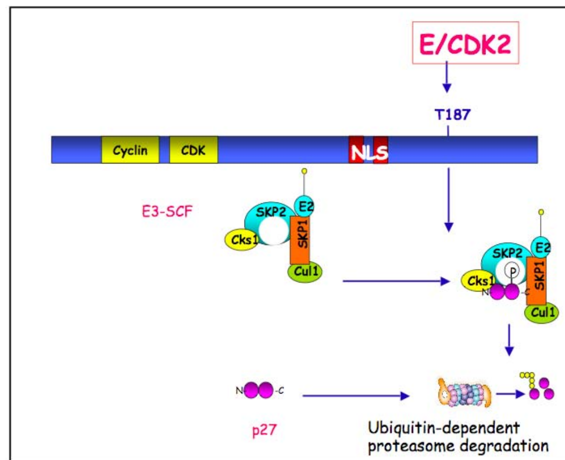


Fig. 4: Molecular mechanims of p27 degradation. Degradation of p27 through the ubiquitin-proteasome pathway is a three-step process that requires: (i) phosphorylation of p27 at threonine 187 by cyclin E/Cdk2; (ii) recognition of T187-phosphorylated p27 by the ubiquitin ligase; (iii) SCF^{Skp2}-dependent ubiquitination and degradation of T187-phosphorylated p27.

Recently, a Skp2-independent pathway for the degradation of p27 at G1 phase has been described (Kamura et al. 2004). Degradation of p27 in G1 occurs after its export from the nucleus to the cytoplasm and the cytoplasmic E3 complex KPC (Kip1 ubiquitination-promoting complex) has shown to be implicated in the Skp2-independent proteolysis of p27 at the G0-G1 transition.

Sequestration by cyclin D-Cdk4/6 complexes is another mechanism whereby p27 activity is regulated during cell cycle (Bouchard et al. 1999; Perez-Roger et al. 1999). Upon mitogenic stimulation, D-type cyclins accumulate in early-to-mid G1, associate with Cdk4/6 and phosphorylate pRB, facilitating further phosphorylation by cyclin E-Cdk2 of pRB and progression along G1. However, formation of cyclin D-Cdk4/6 complexes in mid-G1 serves also to titrate p27 away from cyclin E-Cdk2 complexes (Sherr et al. 1999). This latter function is important since Cdk2 activation requires both

reduction in the abundance of p27 and the titration of residual p27 molecules (Albrecht et al. 1999, Ahamed et al. 2002).

Much less is known about the compartimentalization of p27 during cell cycle progression. To inhibit cyclin E-Cdk2, p27 needs to be imported into the nucleus (Reynisdottir et al. 1997). Nuclear import of p27 depends on the presence of a nuclear localization signal (NLS) localized at the C-terminus of the protein (Zeng et al. 2000). As cells progress along the cell cycle, p27 shuttles between nucleus and cytoplasm. In G0 p27 is almost exclusively nuclear whereas in response to mitogenic stimulation a fraction of p27 is translocated to the cytoplasm (Rodier et al. 2001). The cytoplasmic redistribution of p27 induced by mitogenic stimulation is apparently dependent on the phosphorylation of a specific serine residue (S10) (Rodier et al. 2001). The kinase responsible for S10 phosphorylation has been identified as the human kinase interacting stathmin (hKIS) (Boehm et al. 2002), a nuclear protein that, by phosphorylating p27 on S10 in response to mitogenic stimulation, promotes its nuclear export to the cytoplasm and overcomes p27-induced growth arrest (Boehm et al. 2002). However, recent results using a knock-in mouse model indicated that S10 is not required for p27 cytoplasmic localization (Kotake et al. 2005).

3. p27 deregulation in human cancer: down-regulation or mislocalization?

Cdk inhibitors are potent negative regulators of the cell cycle (Sherr et al. 1999). Thus, pertubations in their activity result in severe disregulation of cell proliferation and failure to suppress tumour growth (Sgambato et al. 2000). The Ink4 Cdk inhibitors are lost through mutation, deletion and/or promoter methylation in a variety of human neoplasms and, in this sense, are true tumor-suppressor genes (Ortega 2002). On the contrary, the Cip/Kip Cdk inhibitor p27 does not fit the classic tumour-suppressor paradigm in humans, since mutations in the p27 gene in human tumors are extremely rare (Kawamata et al. 1995). However, p27 has been defined “tumor suppressor protein” since inactivation of its function has been implicated in the development of human tumor (Blain et al. 2002).

Two different mechanisms have been implicated in p27 inactivation during the process of human carcinogenesis: down-regulation of its expression and exclusion from the nuclear compartment. A drastic reduction in the level of p27 protein (or even a complete loss) is observed in approximatively 50% of human cancer of all types (Slingerland et al. 2000). Reduced p27 expression has been associated with the development of human epithelial tumours originating from the majority of organs, including lung (Esposito et al. 1997), breast (Catzavelos et al. 1997), colon (Loda et al. 1997), ovary (Masciullo et al. 1999), esophagus (Singh et al. 1998), thyroid (Baldassarre et al. 1999) and prostate (Tsihlias et al. 1998). Loss of p27 expression is detected also in a subset of malignancies originating from the central nervous system and from the lymphoid tissue (Erlanson et al. 1998) (Fig.5).

In most human tumors the loss of p27 protein results from altered proteasome-mediated degradation. In fact, comparison of immunoistochemical analysis and in situ hybridization performed on the same biopsies reveals a discordance between the levels of p27 protein and mRNA (Catzavelos et al. 1997; Loda et al. 1997; Cordon-Cardo et al. 1998). Moreover, specimens derived from tumours with low p27 protein levels display enhanced proteasome-dependent degradation of p27 (Esposito et al. 1997; Loda et al. 1997; Piva et al. 1999). Accordingly, an increasing number of studies have implicated the deregulation of Skp2 expression in the down-regulation of p27 expression in cancer (Gstaiger et al. 2001; Chiarle et al. 2002). The reduction in the level of p27 protein contributes to tumor development by allowing an increase in Cdk2 activity and cell proliferation (Catzavelos et al. 1997; Loda et al. 1997; Masciullo et al. 1999).

A finding that is crucial for its clinical implication is that low or absent p27 expression represents an important marker of disease progression in a number of tumor types. In fact, results deriving from the assessment of p27 status in paraffine-embedded slides from patients with primary breast cancer have identified p27 as an independent prognostic factor (Catzavelos et al. 1997; Porter et al. 1997). However, the data are strikingly consistent for a variety of tumor types. In patients affected by lung, colon, esophagus, prostate, bladder, ovarian and oral carcinoma reduced or absent p27 protein levels predicts shorter disease-free and/or overall survival (Esposito et al. 1997; Masciullo et al. 1999; Tsihlias et al. 1998) (Fig.5). Cytoplasmic sequestration of p27 in tumors has been identified only recently as a mechanism whereby cancer cells promote cancerogenesis in humans (Singh et al.1998). Displacement of p27 into the cytoplasm has ben shown to contribute to the anchorage-independent growth of human transformed fibroblasts by maintaining high cyclin-Cdk activity in the nucleus (Orend et al. 1998) and to the increased proliferation associated with the loss of the Tuberous Sclerosis Complex 2 gene product (tuberin), a GTPase activating protein for Rap1a and Rab5 GTPases (Soucek et al. 1998).

The first report showing consistent cytoplasmic localization in human tumors dates back only to 1998, when Ciaparrone and coworkers observed altered p27 localization in approximatively 35% of colon cancer specimens (Ciaparrone et al. 1998). Since then, cytoplasmic sequestration of p27 has been reported for a number of other human malignancies, including prostate (Guo et al. 1997), esophagus (Singh et al. 1998), thyroid (Baldassarre et al. 1999), ovarian (Masciullo et al. 1999) and breast carcinomas (Viglietto et al. 2002; Liang et al. 2002; Shin et al. 2002). Interestingly, cytoplasmic accumulation of p27 is more frequent in well-differentiated tumors whereas loss of expression occurs in more advanced stages.

TABLE 1. Studies showing independent prognostic value of p27

Reference	Site	n	Outcome	Cut off	Outcome (multivariate) P value, RR (CI 95%)[1]
Yatabe et al., 1998	NSC lung	149	OS[2]	<60%, ≥60%	P = 0.037, —
Loda et al., 1997	Colon	149	OS	0, <50%, ≥50%	P = 0.003, RR 2.9 (1.7–5.9)
Palmqvist et al., 1999	Colon	89	OS	Continuous variable	P = 0.01, RR 2.4 (1.2–4.8)
Porter et al., 1997	Breast	278	OS	Low, int, high	P = 0.01, RR 2.7 (1.3–6.0)
Tan et al., 1997	Breast	202	OS	<50%, ≥50%	P = 0.03, RR 3.4 (1.1–10.3)
Catzavelos et al., 1997	Breast	168	DFS[3]	<50%, ≥50%	P = 0.02, RR 2.1 (1.2–4.0)
Tsihlias et al., 1998	Prostate	104	DFS	<25%, ≥25%	P = 0.05, RR 2.1 (1.01–4.3)
Yang et al., 1998	Prostate	89	DFS	<30%, ≥30%	P = 0.02, RR 3.0 (1.2–7.6)
Cote et al., 1998	Prostate	96	OS	0–10%, 11–50%, >50%	P = 0.04, —
Mori et al., 1997	Gastric	138	OS	<50%, ≥50%	P = 0.01, RR 2.6
Newcomb et al., 1999[4]	Ovary	66	OS	<50%, ≥50%	P = 0.02, —
Ito et al., 1999	Hepatocellular	104	DFS	<50%, ≥50%	P = 0.02, —
Erlanson et al., 1998	Lymphoma	105	OS	<15%, ≥15%	P = 0.04, RR 1.9 (1.02–3.44)
Mizumatsu et al., 1999	Astrocytoma	90	OS	<50%, ≥50%	P = 0.002, RR 2.5 (1.4–4.3)
Fan et al., 1999	Laryngeal SCC	109	OS	<10%, ≥10%	P = 0.02, RR 9.8 (1.4–68.6)
Mineta et al., 1999	Tongue SCC	94	OS	<50%, ≥50%	P = 0.02, RR 5.4 (1.3–23.7)

[1]RR = relative risk; CI 95% = 95% confidence interval.
[2]OS = overall survival.
[3]DFS = disease free survival.
[4]Case control study.

Fig. 5: Studies showing p27 expression and its prognostic value in human tumors

In fact, cytoplasmic mislocalization of p27 is more frequent in early (Dukes A and B) and in differentiated (G1-2) colon carcinomas whereas advanced colon cancer shows loss of p27 expression (Sgambato et al. 1999).

Similarly, cytoplasmic accumulation of p27 is observed in well-differentiated thyroid carcinomas while loss of p27 expression is observed in more undifferentiated neoplasms (Baldassarre et al. 1999). In esophageal cancer, cytoplasmic displacement of p27 is more frequent in early displastic lesions than in invasive cancers (Singh et al. 1998). This notwithstanding, it appears that the presence of p27 protein in the cytoplasm of cancer cells is predictive of a more aggressive clinical behaviour, when compared with tumors that present comparable levels of p27 expression (Liang et al. 2002). In fact, patients affected by Barrett's associated adenocarcinomas or by breast carcinomas that present cytoplasmic p27, show decreased overall survival compared with patients who present nuclear p27 (Singh et al. 1998; Shin et al. 2002).

4. Pathways that affect p27 expression and localization

A critical issue in the comprehension of how p27 is inactivated in human cancer is the identification of the intracellular oncogenic pathways that contribute to dysregulate p27 expression and localization. Deregulated signaling from the membrane tyrosine kinase receptors constitutes a hallmark of many cancers (Blume-Jensen et al. 2001). During cancer development,

constitutive activation of the intracellular mitogenic pathways may occur by amplification (as in the case of the EGF receptor, ErbB/HER1 or ErbB2/HER2), chromosomal translocations (as in the case of RET and TRKA) or point mutation (as in the case of RET and MET) of the receptors themselves (Blume-Jensen et al. 2001). Alternatively, mitogenic pathways may be activated at the level of cytoplasmic transductors as in the case of mutations in the three genes of the Ras family, in the case of the deletion of PTEN or of the amplification and/or hyperactivity of PI3K and AKT (Blume-Jensen et al. 2001).

There is now evidence that p27 is a major target of the growth-promoting activity exerted by tyrosine kinase receptors. Constitutive signaling from the ErbB family of tyrosine kinase receptors, including EGFR, ErbB2/Her2 or RET/PTC promotes proliferation of cancer cells by decreasing p27 levels (Yakes et al. 2002; Vitagliano et al. 2004), by sequestrating p27 away from cyclin E-Cdk2 complexes (Lane et al. 2000) or causing its nuclear exclusion. On the other hand, activation of antimitogenic signaling by TGF-β, suppresses cell growth by increasing p27 expression (Slingerland et al. 1994). Down-regulation of p27 protein induced by mitogenic stimulation of tyrosine kinase receptors requires the activity of the GTP-binding protein Ras (Aktas et al. 1997), though the activity of other GTP-binding proteins (RhoA and Ral A) may also be required (Hu et al. 1999; Yamazaki et al. 2001). Oncogenic Ras also mediated nuclear-to-cytoplasmic delocalization of p27, abrogating the ability of TGFβ to inhibit nuclear Cdk2 and to induce growth arrest in mink lung epithelial cells (Liu et al. 2000).

How oncogenic Ras induces down-regulation and mislocalization of p27 is still not completely understood. Ras activates distinct signal transduction pathways, including the phosphatydilinositol-3-kinase (PI3K) pathway and the mitogen-activated-protein kinase (MAP kinase) pathways (Blume-Jensen et al. 2001). Converging data from the recent literature indicate that both PI3K and MAP kinase pathways are involved in the regulation of p27 expression (Fig. 6A and B, respectively).

In fact, p27 down-regulation induced by activated oncogenes (Gesbert 2000) or by mitogens is alleviated by pharmacological inhibitors of the PI3K (i.e. LY294002, wortmannin) or by natural PI3K antagonist (the phosphoinositide phosphatase PTEN) (Bruni et al. 2000; Li et al. 1998). Several mechanisms may account for the effects exerted by the PI3K pathway on p27 expression. First, the PI3K pathway regulates transcription from p27 promoter through AKT-dependent phosphorylation of the forkhead transcription factors Afx and FHKR (Dijkers et al. 2000; Medema et al. 2000); the PI3K pathway regulates the stability of p27 protein through an as yet undefined mechanism (Gesbert et al. 2000). Similarly, down-regulation of p27 protein induced by mitogens or activated ErbB2/Her2, RET/PTC, RAS and RAF requires MAP kinase activation (Aktas et al. 1997; Rivard et al. 1999; Delmas et al. 2001; Vitagliano et al. 2004).

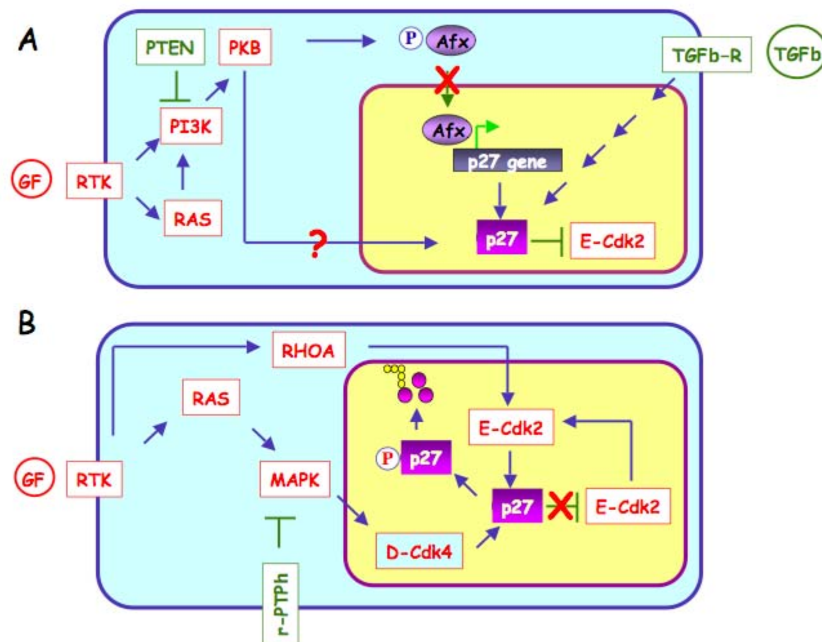


Fig. 6: The different signaling pathways that cause dysregulation of p27 expression and/or localization in human cancer. Activated tyrosine kinase receptors turn on RAS, which in turn activates the PI3K and the MAP kinase pathways. (A) The RAS/PI3K pathway regulates p27 expression by activating Akt, which in turn phosphorylates the forkhead transcription factor Afx, sequestrating it in the cytoplasm and preventing transcription from the p27 gene promoter. Akt also regulates the stability of the p27 protein through an unknown mechanism. (B) The RAS/MAP kinase pathway regulates p27 expression by inducing the formation of cyclin D-Cdk4/6 complexes that sequestrate p27 away from cyclin E-Cdk2 complexes; this allows activation of some cyclin E-Cdk2, that are free to phosphorylate p27 on T187 and target it to destruction in the proteasome. Tyrosine kinase receptors may also degrade p27 through activation of RHOA, which in turn regulates the stability of the p27 protein in a Cdk2-dependent manner. The tyrosine phosphatase rPTPη inhibits RAS-dependent p27 down-regulation by interfering with MAP kinase activation. GF, growth factor; RTK, receptorial tyrosine kinase; PI3K, phosphatydilinositol-3-kinase; MAPK, mitogen-activated-protein kinase.

Different molecular mechanisms account for the MAP kinase-dependent decrease in the intracellular abundance of p27: decreased protein p27 synthesis, decreased p27 protein stability, increased Cdk2 activity or decreased activity of a tyrosine phosphatase (r-PTPη) (Rivard et al. 1999; Trapasso et al. 2000).

As to the control of p27 localization, results from different laboratories suggest that the PI3K/Akt pathway is implicated in the cytoplasmic localization of p27 (Viglietto et al. 2002; Liang et al. 2002; Shin et al. 2002) (Fig. 6A).

5. Cell cycle alterations in human thyroid cancer

Epithelial tumors derived from thyroid gland are usually classified into papillary carcinoma (PTC), follicular carcinoma (FTC) and anaplastic carcinoma (ATC) (Sherman et al. 2003) (Fig. 7). PTC is the most common thyroid malignancy. It shows typical histologycal appearance and is characterised by distinctive nuclear morphology (ground-glass). PTC metastasizes locally via the lymphatics and is usually associated with therapeutic responsiveness and good prognosis. FTC is less frequent than PTC. In many cases FTC retains some aspects of follicle formation, but shows invasion of the capsule and/or blood vessels and metastasizes via the blood, usually to the bone, brain, and lung (Hedinger et al. 1989). ATC is one of the most aggressive solid tumors in humans, showing a mean survival of six months after diagnosis (Giuffrida et al. 2000).
It is becoming apparent that distinct molecular events are associated with specific stages in the multistep thyroid tumorigenic process with good genotype/phenotype correlation. For instance, mutation of the gsp and TSH receptor genes are associated with benign hyperfunctioning thyroid nodules and adenomas while alterations of other specific genes, such as oncogenic kinase alterations (RET/PTC, TRKA, BRAF) in PTC and the newly discovered Pax-8/PPARγ rearrangement in FTC, are the distinctive feature of cancer (Tallini et al. 2001) (Fig. 7). RET/PTC oncogenes are generated by chromosomal rearrangements resulting in the fusion of the RET tyrosine-kinase domain to the 5'-terminal region of heterologous genes (Fusco 1987; Grieco 1990), which results in a chimeric protein that exhibits constitutively active tyrosine kinase activity. RET rearrangements are restricted to the thyroid gland, where they are specific for PTC (Santoro et al. 1992; Santoro et al. 1993), with RET/PTC1 present in approximately 40% of sporadic (i.e. non radiation-associated) PTCs, and RET/PTC3 in about 15% (Tallini et al. 2001). Similar to RET, NTRK1 (or TRKA) oncogene encodes a transmembrane TK receptor for the nerve growth factor (NGF), which is activated in thyroid follicular cells due to chromosomal rearrangements that fuse the NTRK1-TK domain to the 5'-terminal region of different heterologous genes (Pierotti et al. 2001). The TRK oncogenes also appear restricted to papillary thyroid carcinoma but are found with a lower prevalence than that reported for RET/PTC (approximatel 10% of cases (Bongarzone et al. 1998) (Fig. 7).
RAS mutations also play an important role in malignant transformation and progression in thyroid cancer. Constitutive activation of all three RAS oncogenes (K-, N-, and H-RAS) has been identified in tumors originating from the thyroid gland, being more common in FTC than in PTC (Manenti et al. 1994; Shi et al. 1991). RAS proteins exhibit guanosine triphosphatase (GTPase) activity and exist in two forms: an inactive, GDP-linked form and an active GTP-linked one, which functions to convey signals originating from tyrosine kinase membrane receptors to a cascade of mitogen-activated phosphokinases (MAPK), resulting in cell proliferation. Further downstream, mutations in the BRAF gene, which encodes a serine-threonine kinase involved in the transmission of signals from membrane receptors and RAS small

GTPases to MAPK, have been implicated in the carcinogenesis of PTCs (Fig. 7). Several groups have reported that about 50% of adult PTCs harbor a specific point mutation (V600E) (), which activates constitutively the BRAF kinase (Dibb et al. 2004).
Another typical alteration frequently observed in thyroid cancer is the constitutive activation of PI3K/Akt pathway. In fact, PI3KCA gene amplification has been recently detected (in FTAs and FTCs) (Wu et al. 2005), whereas conflicting results have been reported on the presence of activating mutations of the PIK3CA gene in thyroid carcinomas (Wu et al. 2005). Furthermore, recent studies have shown increased expression of AKT in FTCs (Ringel et al. 2001), PTCs (Vasko et al. 2004) and of p70S6 Kinase and Akt in the majority of PTCs (Miyakawa et al. 2003). Mutations in the PTEN gene or loss of 10q22-23 chromosomal region (the PTEN locus) have been identified in approximately one fourth of sporadic FTAs and FTCs, but rarely in PTCs (Halachmi 1998; Dahia 1997; Bruni 2000). However, since decreased PTEN protein levels are frequently detected in thyroid cancer (Bruni et al. 2000; Gimm et al. 2000), it is likely that mechanisms other than gene inactivation by mutation may be responsible for the reduced PTEN function in thyroid tumors.

Both the MAP kinase and the PI3K/Akt pathways transduce mitogenic signals in response to the activation of RAS and tyrosine kinase receptors (Blume-Jensen et al. 2001). In the end, these pathways impinge directly on cell cycle regulators. Accordingly, oncogenic RAS induces $p27^{kip1}$ loss in human normal thyrocytes (Jones et al. 2000); activated RET/PTC induces MAP kinase dependent degradation of $p27^{kip1}$ expression in rat and human thyroid cells, whose expression is restored upon pharmacological inhibition of endogenous or transfected RET/PTC (Vitagliano et al. 2005). In addition to these indirect dysregulation of cell cycle regulators induced by more upstream molecular lesions, over-expression of the positive regulators (cyclins) or under-expression of the negative regulators (Cdk inhibitors) of cell cycle has been also documented to occur in thyroid neoplasms. In fact, overexpression of cyclin D1 and E has been shown in several types of thyroid tumors (Wang et al. 1999). Among negative regulators, p53 gene mutations are restricted to poorly- and undifferentiated tumors (Donghi et al. 1993; Dobashi et al. 1994), whereas no significant abnormalities of the Rb gene are believed to occur in either benign or tumors malignant thyroid neoplasms (Holm et al. 1994). The Cdk inhibitors p15 and p16 are rarely mutated in primary thyroid (Hirama et al. 1995), though the finding of hypermethylation of the p16 gene promoter have suggested that functional inactivation of p16 can occur as an epigenetic event during thyroid tumorigenesis (Elisei et al. 1998). By contrast, deletions of the Cdk inhibitor p21 gene have been reported in approximately 10% of PTCs (Shi et al. 1996). Finally, p27 is expressed in normal thyroid follicular cells but its expression is markedly decreased in roughly half of carcinomas, due to increased protein degradation (Erickson et al. 1998; Baldassarre et al. 1999). Loss of p27 correlates with aggressive, high-grade tumors and poor prognosis. Nonetheless, certain carcinomas of the thyroid contain normal or

even increased levels of p27 but, strangely, the protein has shifted location in these cancers (Baldassarre et al. 1999). Normally p27 resides in the nucleus, but in these tumors it can be found instead in the cytoplasm. My Doctorate thesis will be focused to investigate the molecular mechanisms whereby p27 is relocalized to cytoplasm in thyroid cancer cells and to determine the functional meaning of such delocalization.

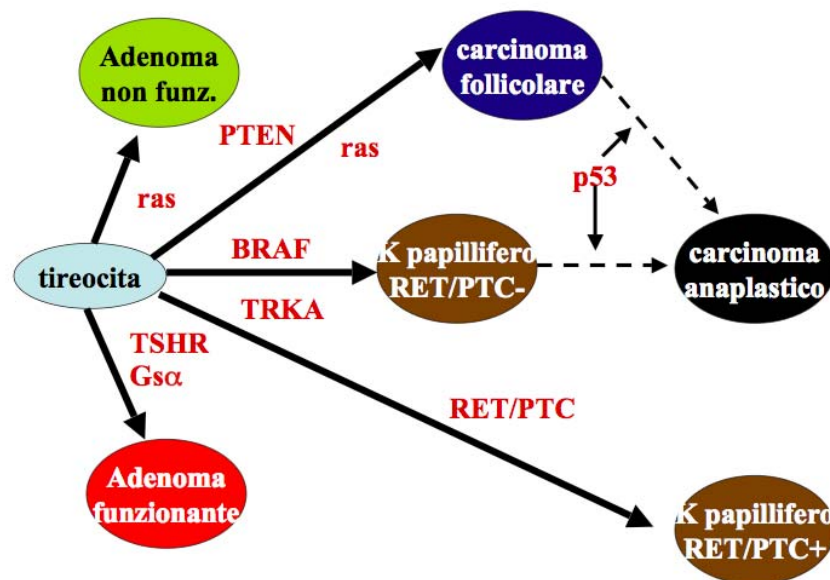


Fig.7: Molecular genetics of thyroid cancer.

6. The serine-threonine Akt

The Akt gene product is the cellular homolog of the v-Akt oncogene transduced by AKT8, an acute transforming retrovirus in mice (Staal et al. 1987). In 1991, three independent research groups cloned and characterized Akt kinase. The group of Philip Tsichlis identified v-Akt as the gene transduced by rodent retrovirus AKT8 (Bellacosa et al. 1991), and subsequently showed that its cellular homolog, then named c-Akt encoded the cytoplasmic serine–threonine protein kinase Akt (Bellacosa et al. 1993). Akt was also identified in a search for novel kinases related to protein kinases A and C as protein kinase B (PKB, as a kinase similar to protein kinases A and C), and RAC-PK (kinase related to protein kinases A and C) (Coffer et al. 1991).

In mammals three closely related isoforms of Akt are encoded by distinct genetic loci: Akt1 (that in fact is Akt), Akt2 and Akt3 (Fig. 7)

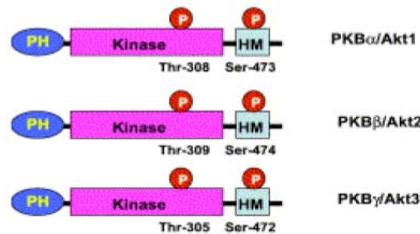


Fig.7: Structure of the members of Akt family. PH, Pleckstrin Homology domain; Kinase, catalityc domain.

All Akt serine-threonine kinases share a common structure that consists of an N-terminal regulatory pleckstrin homology (PH) domain (Franke et al. 1994), a hinge region, and a kinase domain with serine-threonine specificity (Ahmed et al. 1993; Chan et al. 1999) (Fig. 7). The PH domain, so called because it was originally found in pleckstrin, interacts with membrane lipid products such as phosphatidylinositol-(3,4,5)trisphosphate (PIP3) and phosphatidylinositol-(3,4)disphosphate (PIP2) produced by PI3K, a lipid kinase that phosphorylates phosphoinositides on the 3'-OH position of the inositol ring (Rameh et al. 1999). Biochemical analysis revealed that the PH domain of Akt binds to both PIP3 and PIP2 with similar affinity (Frech et al. 1997; James et al. 1996). The kinase domain of Akt, located in the central region of the molecule, shares a high similarity with other AGC kinases such as PKA, PKC, p70S6K and p90RSK (Peterson et al. 1999).
A number of stimuli can promote activation of Akt through the activation of receptor tyrosine kinases such as platelet derived growth factor receptor (PDGF-R), insulin, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor I (IGF-I) (Fig. 8). The finding that Akt activation by receptor tyrosine kinases is blocked by the PI3K inhibitors wortmannin and LY294002 indicates that PI3K activity is necessary for Akt activation (Chan et al. 1999; Chan et al. 2003).

The exact mechanism of Akt activation by PI3K has been studied thoroughly, and requires two consecutive events: relocalization to plasma membrane and phosphorylation of two key residues (T308, S473) that allow full activation of the kinase domain (Alessi et al. 1996). The PI3K-dependent activation of Akt is initiated by the binding of Akt PH domain to PIP2 and PIP3, inducing the relocalization of the cytoplasmic Akt protein to signaling complexes at the plasma membrane (Franke et al. 1997; Frech et al. 1997). Oncogenic mutations of Akt that result in a constitutive activation include constitutive plasma membrane binding by N-terminal myristoylation (Franke et al. 1995; Meier et al. 1997). Full activation of membrane-bound Akt requires the phosphorylation of two conserved residues, T308 and S473 (Alessi et al. 1996).

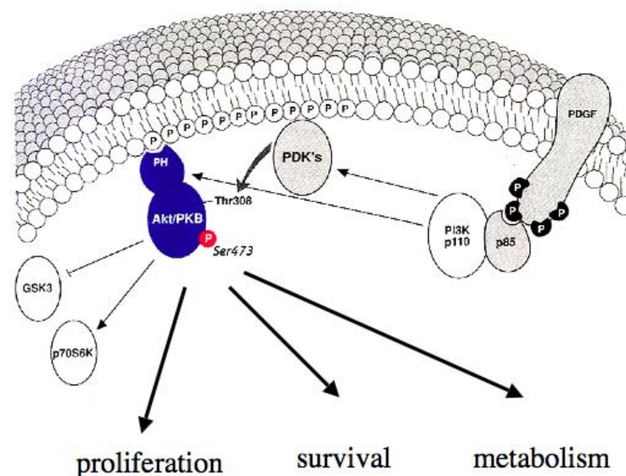


Fig. 8: The PI3K/Akt pathway.

The T308 residue is located in the activation segment, whose phosphorylation is required for substrate peptide recognition and transfer of phosphate (Huse et al. 2002), whereas S473 is located in the "activation loop", a carboxyl terminal domain of 40 amino acids that possess an hydrophobic motif characteristic of all members of the AGC kinase family (Peterson et al. 1999). The kinase responsible for T308 phosphorylation is PDK1 (Alessi et al. 1997; Stephens et al. 1998). It has a PH domain at its carboxyl-terminal, indicating that translocation to the plasma membrane is necessary for its full activation (Andjelkovic et al. 1997, Anderson et al. 1998). The identity of S473 kinase is much more controversial. Recently, the target of rapamycin (TOR) kinase and its associated protein rictor have been shown to directly phosphorylate Akt on S473 in vitro and to facilitate

T308 phosphorylation by PDK1 (Sarbassov et al. 2005). Once initiated, the signaling induced by PI3K is terminated by phosphoinositide-specific phospholipid phosphatases such as PTEN or SH2-domain-containing inositol phosphatases that dephosphorylate PIP3 at the 5' position (SHIPs).

7. Physiological functions of Akt

Akt isoforms contribute to a variety of cellular responses, including cell growth, survival and metabolism. This multiplicity of Akt functions might be due to the variety and specificity of its substrates. The minimal substrate consensus sequence for Akt, RXRXXS/T, where X is any amino acid and S/T is the phosphorylation site, is based on the sequence around the phosphorylation site on GSK3, the first identified substrate (Datta et al. 1999; Brazil et al. 2001). So far at least a dozen of Akt substrates has been identified. As to the effects exerted by Akt phosphorylation on its substrates, it may either increase or decrease the activity of substrates with intrinsic enzymatic activity, or may increase the affinity of the substrate(s) for interaction with 14-3-3 proteins, a class of proteins that are abundantly expressed in the cytoplasm and specifically bind phosphoserine-threonine-containing polypeptides, thus retaining phosphorylated Akt substrates in the cytosol (Franke et al. 1997).

7.a. Akt substrates with metabolic functions

GSK3, a kinase responsible for the inactivation glycogen synthase in response to insulin, was the first physiological substrate identified for Akt (Burgering et al. 1995). Both isoforms (GSK3α and β) have the phosphorylation site in the amino-terminal region (S21 and S9, respectively), and they are phosphorylated and inactivated by Akt. Also the cardiac-specific isoform of 6-phosphofructo-2-kinase (6-PF2-K) is phosphorylated by Akt on S466, which results in the activation of this enzyme and promotion of glycolysis (Deprez et al. 1997). AKT can phosphorylate also the mammalian target of rapamycin (mTOR) (Nave et al. 1999; Scott et al. 1998), a regulator for mRNA translation in the signaling pathway controlled by nutrients, is another Akt substrate on S2448 to activate it (Schmelzle et al. 2000); and TSC2 (the products of the tumor suppressor genes tuberous sclerosis 2 that is responsible for tuberous sclerosis) on S939 and T1462 (Krymskaya et al. 2003), resulting in suppression of the S6 kinase inhibiting potential of TSC2 (Inoki et al. 2002; Manning et al. 2002) and in TSC2 degradation (Dan et al. 2002).

7.b Substrates of Akt in the regulation of apoptosis

An important function of activated PI3K in cells is the inhibition of programmed cell death and Akt is a good candidate for mediating it (Datta et al. 1999). BAD (Bcl-2/Bcl-X antagonist) is a member of the Bcl-2 family of proteins that binds Bcl-2 and Bcl-X and inhibits their anti-apoptotic potential (Downward et al. 1999). When BAD is phosphorylated on S136 by Akt, it does not exhibit proapoptotic activity (Del Peso et al. 1997; Datta et al. 1997). In fact, once phosphorylated, BAD is released from a complex with Bcl-2/Bcl-X that is localized on the mitochondrial membrane, and forms a complex with 14-3-3). Akt can also phosphorylate pro-caspase 9, an initiator and an effector of apoptosis, on

S196 (Cardone et al. 1998) and inhibits cytochrome C-induced cleavage of this pro-caspase 9, which is required for enzymatic activity (Donepudi et al. 2002). Akt may regulate apoptosis also through the phosphorylation of transcription factors that are responsible for pro- as well as anti-apoptotic genes. The role of Akt in the signaling pathway regulating the Forkhead (FH or FoxO) family of transcription factors was first identified by findings from the genetic analysis of C. elegans (Paradis et al. 1998; Paradis et al. 1999) To date, four isoforms of FH proteins (FKHR/FoxO1, FoxO2, FKHRL1/FoxO3 and AFX/FoxO4) are phosphorylated by Akt directly (Rena et al. 1999; Kops et al. 1999). Phosphorylation sites for Akt are highly conserved among FH isoforms and species: they are in human FKHR/FoxO1, T21, S256 and S318. Phosphorylation of FH by Akt results in the exclusion of FH from cell nuclei, leading to decreased transcriptional activity that is required for promoting apoptosis.

7.c Akt-dependent control of cell cycle progression

The cyclin/Cdk inhibitor p21, which plays a crucial role in regulating cell cycle progression, is reported to be a direct substrate for Akt, and phosphorylation of this protein results in the inhibition of its potential to arrest the cell cycle (Zhou et al. 2001A) Phosphorylation of p21 on T145 by Akt can inhibit nuclear localization of p21, leading to activation of cyclin/CDK required for HER-2/neu-dependent tumor cell growth or prevent formation of the complex between p21 and proliferating cell nuclear antigen (PCNA) that causes DNA replication and cell proliferation (Rossig et al. 2001). Another report (Li et al. 2002) showed that p21 can be phosphorylated on S146 as well as T145; and that phosphorylation of T145 reduces affinity to PCNA, whereas phosphorylation of S146 enhances protein stability of p21. The ubiquitin E3 ligase MDM2, an oncogene that directly binds to p53 and targets it for ubiquitination, is another Akt substrate (Shimizu et al. 2003). In 2001, two groups reported that Akt-dependent phosphorylation might contribute to nuclear localization of MDM2 (Mayo et al. 2001; Zhou et al. 2001B). Two putative phosphorylation sites, S166 and S186, were identified (Ashcroft et al. 2002). However, the precise effect of Akt-dependent phosphorylation on MDM2 is still controversial (Ogawara et al. 2002).

Aims of the study.

The cyclin-dependent kinase inhibitor p27 is an inhibitor of the cell cycle, and thus a candidate tumor suppressor by birthright. But the homozygous loss or silencing of the p27 locus is extremely rare. Nonetheless, a decrease in p27 levels, due to p27 protein degradation occurs in roughly half of carcinomas and correlates with aggressive, high-grade tumors and poor prognosis. However, certain carcinomas of the breast, thyroid, esophagus or colon contain normal levels of p27 but, strangely, the protein has shifted location in these cancers. Normally p27 resides in the nucleus, but in these tumors it can be found instead in the cytoplasm. How does this happen? And, most important, what does it mean?
To address these issues, we have chosen to study tumors derived from thyroid follicular cells as a model system since inactivation of p27 in these neoplasms occurs both through loss of expression and mislocalization.

On this basis the main objectives of my doctorate thesis were:

(1) To identify the molecular mechanisms involved in cytoplasmic relocalization of p27 in cancer cells, with particular attention to identifying novel p27 kinases. In particular, my interest was focused to determine whether p27 represents a novel Akt substrate, to identify the putative phosphorylation sites, and to determine the biochemical and biological effects of such phosphorylations.
(2) To determine the role of cytoplasmic localization of p27 in normal and pathological cell cycle, defining the role of p27 protein phosphorylation in this process.
(3) To perform a sistematic investigation of the expression and subcellular localization of p27 in cell lines and primary tumors derived from benign (adenomas) and malignant (PTC, FTC, ATC) thyroid tumors.
(4) To correlate the expression and subcellular localization of p27 observed in primary tumors derived from benign (adenomas) and malignant (PTC, FTC, ATC) thyroid tumors with the status of activation of thye PI3K/Akt pathway.

Materials and Methods.

Cell lines and reagents.

The human thyroid carcinoma cell lines used in this study were: TPC-1, NPA, (derived from PTC), WRO (derived from FTC), FRO and FB1 (derived from ATC) (see Baldassarre et al. 1999 for references). The HEK293 and MCF7 human cell lines were from the American Tissue Cell Culture collection. All cell lines were grown in Dulbecco's modified eagle medium (DMEM) containing 10% foetal calf serum.

Tissue specimens.

Thyroid carcinomas were collected from the Chirurgia B of National Cancer Institute “Fondazione G Pascale”, Napoli, Italy or retrieved from the files of the Department of Scienze Biomorfologiche e Funzionali at the University Federico II of Naples. Diagnosis was based on standard histological critera. Special care was taken to select paraffin blocks without oxyphilic (Hurtle) changes, being these source of aspecific cytoplasmatic staining, and including both the tumor and the rim of normal thyroid tissue around it, the latter to serve as control of the immunohistochemical staining.

Immunostaining.

Serial sections were stained using monoclonal anti-p27 antibody (Transduction Laboratories, Lexington, KY, USA) (dilution 1:4000), the polyclonal antibody anti-phospho-AKT (Ser473) from Cell Signaling Technology (dilution 1:250). Incubation with primary antibodies, was followed by incubation with biotinylated anti-mouse/rabbit immunoglobulins, and by peroxidase labeled streptavidine (LSAB-DAKO). Signal was developed with diaminobenzidine as chromogen. Incubations omitting the specific antibody, as well as with unrelated antibodies, were used as controls of the technique. Specificity of the antibodies was assessed by competition with antigens used for the antibody production (full length p27, phospho-AKT blocking peptides, respectively). Tumors were scored as p27 positive or p27 negative on the basis of a staining cut-off of 50% as described (Baldassarre et al. 1999). Both nuclear and cytoplasmatic staining was considered to evaluate p27 expression. P27 positive tumors with both cytoplasmatic and nuclear or cytoplasmic staining were scored as “cytoplasmatic”, whereas tumors with exclusive nuclear staining were scored as “nuclear”. The cut-off value to score tumors positive for AKT activity was 10% of positive cells.

Statistical analysis

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

BrdU incorporation and indirect Immunofluorescence

5-Bromo-2'deoxyuridine-5'-monophosphate (BrdU) incorporation assay was performed as follows: cells were grown to subconfluence on coverslips, incubated with 10 μM BrdU for 2 hours and then fixed in 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. BrdU-positive cells were visualized using Texas-Red-conjugated secondary antibodies (Baldassarre et al. 1999; Bruni et al. 2000). P27-positive cells were evidentiated using FITC-conjugated secondary antibodies. Cell nuclei were identified by Hoechst staining. Fluorescence was visualized with Zeiss 140 epifluorescent microscope equipped with filters that discriminated between Texas Red and fluorescein. All assays were performed 3 times in duplicate.

Flow cytometry

Cell cycle distribution was analyzed by flow cytometry as described previously (Baldassarre 1999). In brief, cells were harvested in phosphate-buffered saline (PBS) containing 2 m*M* EDTA, washed once with PBS, and fixed for 30 min 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 min. They were then washed once in PBS and stained with 50 μg/ml propidium iodide (Roche, Basel, Switzerland). Stained cells were analyzed with a fluorescence-activated cell sorter (Becton-Dickinson), and the data were analyzed using a Mod-Fit cell cycle analysis program.

Peptide Synthesis and Pull-downs

Biotinylated peptides were synthesized on a solid-phase peptide synthesizer using amide resin (Neosystem, France). Amino acid sequences of the biotinylated peptides used were: YRKRPATDDSSTQNKR (157); YRKRPAT(P)DDSSTQNKR (157); SVEQTPKKPGLRRRQT(198); SVEQTPKKPGLRRRQT(P)(198). Peptides were designed as 16-mers bearing an N-terminal biotin moiety. The identity and purity of the synthesized peptides were confirmed by mass spectrometric analysis. Peptides were synthesized as pairs as "phosphorylated" and "non-phosphorylated" forms. For affinity pull-downs, 5 μg of immobilized peptide was added to 250 μg of cell lysate from TPC-1, NPA, WRO, ARO, FB-1 cells.

Immobilized streptavidin beads (Pierce) were loaded with biotinylated peptides at 4°C overnight prior to incubation with cell lysates. Cells were. Equal amounts of proteins prepared in NP-40 extraction buffer [1% (v/v) Nonidet P-40, 150 mM sodium chloride, 50 mM Tris-HCl pH 7.5], were incubated with the respective immobilized peptides at 4 °C for 1 hour. After extensive washes, bound proteins were eluted from the immobilized peptides by boiling in SDS sample buffer.

Protein extraction, western blotting and antibodies.

Total proteic extracts were obtained in NP-40 extraction buffer as described (Baldassarre 1999). Nuclear and cytoplasmic proteins were differentially extracted by lysing cells in ice-cold hypotonic buffer (0.2% NP-40, 10 mM Hepes pH 7.9, 1 mM EDTA, 60 mM KCl). Nuclei were separated through a 30% sucrose cushion and lysed in hypertonic buffer (250 mM Tris-HCl pH 7.8, 60 mM HCl). Antibodies to SP1 and γ-tubulin were used to assess the purity of the fractions. Immunoblots were performed as described (Baldassarre 1999; Bruni 2000).

The antibodies used in these studies were: anti-HA (Roche); anti-FLAG (M5; Sigma, St. Louis, MO); anti-CDK2 (M2) (Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-p27 (Transduction Laboratories, Lexington, KY); anti-Akt and anti-phosphoAkt (Ser473) (New England Biolabs, Lake Placid, NY); phospho-(Ser/Thr) Akt substrate antibody, raised against the Akt consensus phosphorylation sequence (RXRXXT/S) (#9619, Cell Signaling Technology Inc., Beverly MA, USA). The Phospho-specific antibodies (P-T157 and P-T198) were obtained by immunizing rabbits against the peptides containing P-T157 or P-T198, respectively (see Peptide Synthesis and Pull-downs section for sequences) and purified through sequential passage on columns linked to the unphosphorylated peptides followed by a final purification on columns linked to the phosphorylated peptides.

Gel overlay assay

Gel overlay assay was performed using recombinant 14.3.3ε as the ligand. Briefly, 6HIS-tagged wild type and mutant p27 proteins (approx. 1 μg) were phosphorylated in vitro by recombinant Akt, separated by SDS-PAGE (12.5%) under reducing conditions, and transferred onto nitrocellulose membrane. The nitrocellulose paper was blocked by a 45 min incubation with 5% non-fat milk (w/v) in TTBS [0.02M Tris/HCl/0.5 M NaCl, pH 7.5, containing 0.2% (v/v) Tween-20] and incubated for 3 hours with 1 mg/ml V5-tagged 14.3.3ε in 5% BSA and 50 mM imidazole. Subsequently, membranes were incubated with HRP-linked anti-V5 antibody and revealed by enhanced chemioluminescence.

Immunoprecipitation

Cells were lysed in 1% (v/v) Nonidet P-40, 150 mM sodium chloride, 50 mM Tris-HCl, pH 7.5, 1 mM sodium orthovanadate, and protease inhibitors (Roche Applied Science) and incubated at 4 °C with a rabbit antibody against Sos isoforms (Santa Cruz Biotechnology). Protein A-Sepharose beads were added after 1 hour and incubated for additional 1 hours. After extensive washes, co-precipitated proteins were eluted in sample buffer for SDS-gel electrophoresis.

Preparation of recombinant p27 and *in vitro* kinase assays.

The cDNA encoding human p27 was cloned into the pET21a vector (Novagen Inc., Madison, WI) in-frame with the hexahistidine tag at the C-terminus. Recombinant p27 was purified using Ni-NTA resin (Quiagen GmbH,

Germany) as described (Polyak 1994). Akt immune complexes were prepared by immunoprecipitation with anti-HA antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and collected on protein A-Sepharose beads and incubated as described (Datta 1997). Recombinant Akt was from Upstate Biotechnology (Lake Placid, NY). The phosphorylated substrates were separated on 12.5% SDS-PAGE and quantified by PhosphorImager (GS525, Biorad, Hercules, CA).

***In vivo* labelling of cells**.

Transiently transfected HEK293 cells were incubated overnight in phosphate-free medium supplemented with 10% dialyzed foetal calf serum and subsequently metabolically labelled with 1 mCi/ml ^{32}P-ortho-phosphate (Amersham Pharmacia Biotech, Little Chalfont, UK) for 4 h at 37°C. Proteins were immunoprecipitated with specific antibodies or with preimmune mouse or rabbit serum, fractionated onto polyacrylamide SDS gel, transferred to nitrocellulose filters and subjected to autoradiography. The incorporated radioactive phosphate was determined by PhosphorImager analysis and normalized by the abundance of p27 in the immunoprecipitates.

Constructs and Transfections.

Akt constructs were described by Bellacosa and coworkers (Bellacosa 1998). The wild type p27 construct has been described previously (Baldassarre 1999; Bruni 2000). The p27-T157A, p27-T198A and p27-T157A/T198A mutants were generated with a site-specific mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. Cells were transfected with Fugene 6 (Roche, Basel, Switzerland). Average transfection efficiency into HEK293 cells was about 80-90%.

Results and Discussion

1. *Akt is a 'bona fide' p27 kinase*

In this wok, we provide experimental evidence that p27 is an Akt substrate. A minimal consensus motif has been defined for AKT (Obata 2000) and is shown in figure 9. Analysis of the human p27 protein sequence revealed 2 threonine residues, T157 (152-RKRPAT-157) and T198 (194-LRRRQT-198), which match the minimal consensus for Akt-mediated phosphorylation (RxRxxT/S). T157 is located within the nuclear localization signal (residues 153-169) whereas T198 represents the last amino acid of the protein.

To determine whether p27 protein is a *bona fidae* Akt substrate we performed an *in vitro* kinase assay (for further experimental details see Viglietto et al. 2002; Motti et al. 2004). Constitutively active Akt was either purchased from Upstate Biotechnology or recovered by immunoprecipitation with anti-HA antibodies, from HEK293 cells transiently transfected with a plasmid encoding a myristoylated, constitutively active, version of Akt (myrAkt). The recombinant p27, used as substrate in the kinase assay, was a 6-His-tagged protein bacterially produced and purified on NiNTA beads. As shown in figure 9, constitutively active Akt (hereafter referred to as ca-Akt) phosphorylated recombinant p27 protein in an immunocomplex kinase assay, whereas a kinase-dead form of Akt (Akt-K179M) did not.

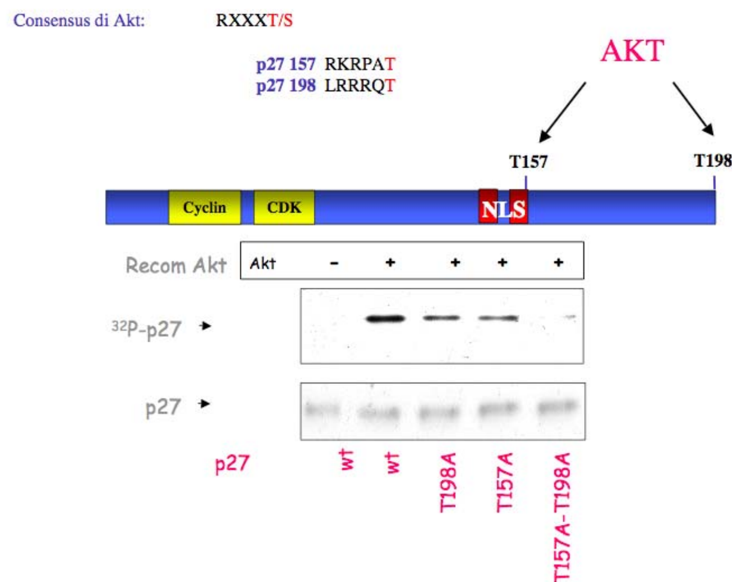


Fig.9: Akt phosphorylates p27. Recombinant 6his-tagged wilt-type (1μg) (lanes 1 and 2) or mutant T157A, T198A, T157A-T198A p27 (lanes 3-5, respectively) proteins were incubated with constitutively active Akt (lanes 2-5) in presence of ^{32}P-γ-ATP. To normalize for the amount of p27 blots were probed with anti-p27 antibodies.

We also identified the sites of Akt-mediated phosphorylation by generating three different mutants of p27 in which T157 and T198 were replaced by non-phosphorylable alanine residues (T157A, T198A single mutants, and T157A-T198A double mutant). P27-T157A was less efficiently phosphorylated by ca-Akt (40% of the original phosphorylation) compared with the wild type p27; the p27-T198A mutant was phosphorylated by ca-Akt at about 50% compared with the wild-type; the simultaneous presence of both the T157 and T198 mutations into the p27 protein completely abolished Akt-dependent phosphorylation (Fig. 9). These results demonstrate that T157 and T198 represent the unique, direct Akt phosphorylation sites. The demonstration of Akt-dependent *in vivo* phosphorylation of p27 on T157 and T198 was obtained by metabolic labeling of cells with ^{32}P-ortho-phosphate (Viglietto et al. 2002; Motti et al. 2004), and will not be described in detail here.

To study phosphorylation of endogenous p27^{kip1} in cancer cell lines and primary tumours, we generated two affinity-purified polyclonal antibodies able to discriminate phosphorylated T157 and T198 from the corresponding unphosphorylated residues (anti-P-T157 and anti-P-T198 antibodies, respectively). The anti-phospho antibodies were tested in western blot experiments in both *in vitro* kinase assays and in *in vivo* transfection experiments (Fig. 10). HA-tagged wild type and mutant p27 were transiently transfected in HEK293 cells together with an excess of ca-Akt or empty control pCDNA3 vector. P27 was recovered by immunoprecipitation and analysed with phospho-specific antibodies. Results from these experiments

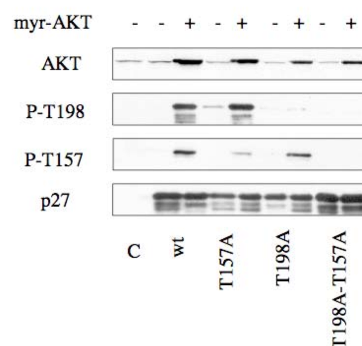


Fig. 10: Akt phosphorylates p27. Plasmids (1μg) encoding HA-tagged wild type or mutant T157A, T198A, T157A-T198A p27 were transfected into HEK293 cells with myrAkt (10μg). After 48 hours cells were harvested and lysed. Proteins (50μg) were separated by SDS-PAGE, transferred onto nitrocellulose filters and decorated with anti-phospho-T198 and anti-phospho-T157 antibodies. The reactivity of phospho-specific antibodies was abrogated by treatment with calf intestinal phosphatase (CIP) (see Viglietto et al., 2002; Motti et al., 2004).

are shown in figure 10 and indicated that the anti-phospho-T157 and anti-phospho-T198 antibodies reacted with p27 only when it was pre-incubated with ca-Akt. The anti-phospho-T198 antibody reacted exclusively with wild type HA-p27 and HA-p27-T157A but not with HA-p27-T198A (only in the presence of ca-Akt); conversely, the signal recognized by the anti-phospho-T157 antibody was greatly reduced in the HA-p27-T157A and HA-p27-T157A-T198A mutants (see Viglietto et al., 2002; Motti et al., 2004).

Altogether these results indicate that recombinant, transfected or endogenous (from IGF1-stimulated human breast cancer cells) Akt phosphorylated p27 *in vitro* and *in vivo* at both T157 and T198 residues.

2. Akt physically associates with p27.

Subsequently, we determined that Akt and p27 proteins form a complex that can be immunoprecipitated from intact cells. This interaction takes place in the cytoplasmic compartment of MCF-7 cells, which contains most of the Akt protein, and is apparently independent of Akt kinase activity, unlike phosphorylation-dependent interaction between Akt and p21 (Zhou *et al.*, 2001). Interaction between Akt and p27 was investigateded by co-immunoprecipitation experiments in HEK293 cells transfected with HA-tagged ca-Akt and FLAG-tagged p27. Western blot experiments with anti-HA on anti-FLAG immunoprecipitates (and viceversa) demonstrated that Akt co-immunoprecipitates with p27 (Fig. 11A). Interestingly, neither T157 nor T198 were required for such interaction. The finding that p27 coimmunoprecipitates with Akt-K179M (Fig. 11B) indicates that the p27-Akt interaction is independent of Akt kinase activity. Subsequently, we used the thyroid cancer cell line NPA cells to determine whether the interaction between Akt and p27 can occur at the endogenous level of expression, and to establish the subcellular location of the interaction. Experiments of co-immunoprecipitation performed in cytoplasmic and nuclear lysates, demonstrate that the Akt-p27 complexes were present only in the cytosolic compartment (Fig. 11C).

3. Cytosolic relocalization of exogenous p27 upon Akt-mediated phosphorylation.

The regulation of subcellular localization is emerging as a major mechanism that govern several cellular processes, such as apoptosis and cell cycle progression (Reynisdottir et al. 1997, Baldassarre et al. 1999; Zhou et al. 2001). Although, the mechanisms that control the compartimentalization of

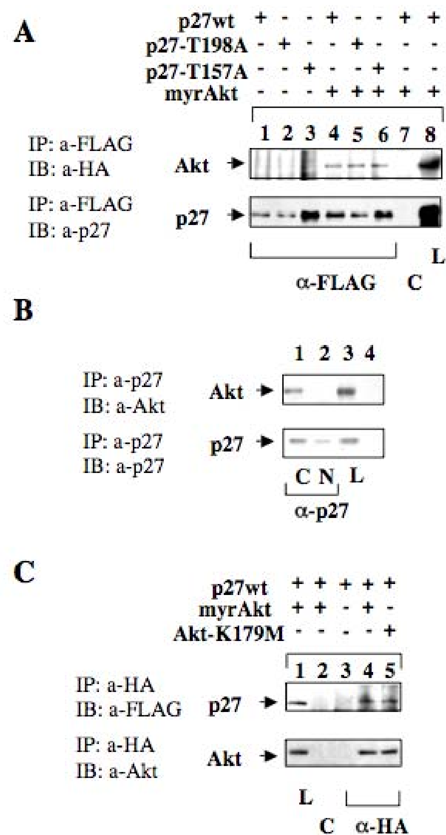


Fig. 11: Physical association between Akt and p27^{kip1}. (A) Anti-HA immunoblot of anti-FLAG immunoprecipitates from HEK293 cells co-transfected with myrAkt and different FLAG-tagged p27^{kip1} constructs, as indicated. Immunoprecipitates were normalized by immunoblot with anti-p27^{kip1}. (B) Anti-FLAG or anti-Akt immunoblotting of anti-HA immunoprecipitates from HEK293 cells transfected with myrAkt or Akt-K179M. L: 50 µg of protein lysate. C: non-immune mouse serum. (C) Cytoplasmic (C) and nuclear (N) proteins were prepared from NPA were immunoprecipitated with anti-p27^{kip1} and immunoblotted as indicated.

proteins are different and still largely unknown, reversible phopshorylation of proteins on serine and threonine residues is believed to play a fundamental role in the processes of nucleus-cytoplasmic shuttling. Among others, the protein

kinase Akt regulates, in a phosphorylation-dependent manner, the subcellular localization of several proteins such as BAD, the FH transcription factors, mdm2, the cyclin-dependent kinase inhibitor p21 (Zhou et al. 2001). On this basis, we determined whether Akt-dependent phosphorylation of p27 affected its cellular localization, and determined the relative importance of the two different Akt phosphorylation sites. HA-tagged, wild type or mutant p27 were transfected into HEK293 cells along with an excess of ca-Akt and analysed by indirect immunofluorescence. The average results of 4 independent experiments are shown in the bar-graph of Figure 12A.

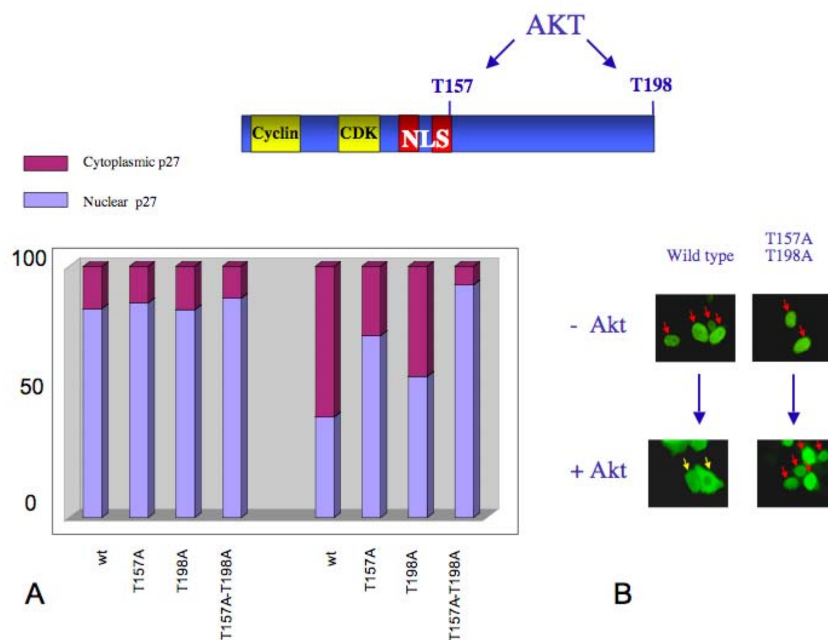


Fig. 12: Akt promotes cytosolic localization of p27. (A) ca-Akt (10µg) was cotransfected with FLAG-tagged wild type p27 or T157A, T198A and T157A-T198A mutants (1µg) in HEK293 cells. After 36 h cells were plated onto coverslips and processed for immunofluorescence. P27 was detected with anti-FLAG monoclonal antibodies and revealed with Fluorescein-linked immunoglobulins. The results are the average of 4 experiments in which at least 500 transfected cells were counted. (B) A representative experiment: red arrows indicate nuclear p27, yellow arrows indicate cytoplasmic p27.

As expected, wild type or mutant p27 co-expressed with the control vector localized almost entirely to the nucleus (~20% of cells showed cytoplasmic staining). In the presence of ca-Akt, nuclear p27 expression was reduced, being found in the cytoplasm in about 60% of transfected HEK293 cells. At difference with the wild-type protein, fewer HEK293 cells presented cytoplasmic p27 when transfected with the HA-p27-T157A (32.6%) or HA-p27-T198A (43.8%) mutants in presence of ca-Akt, whereas the number of cells showing cytoplasmic p27 was almost abolished only when transfected

with the double HA-p27-T157A-T198A mutant (7.6%). A representative immunofluorescence experiment is shown in Figure 12B.

From these results we concluded that Akt-mediated phosphorylation is mechanistically linked to the regulation of the subcellular distribution of p27. Non-phosphorylated p27 was predominantly localized in the nucleus; upon phosphorylation of T157 and T198 residues most of the protein remained in the cytoplasm. Apparently, T157 phosphorylation of p27 is more important than T198. The data are consistent with the possibility that binding to 14-3-3 contributes to the cytosolic localization of T157-phosphorylated p27.

4. Akt rescues p27-mediated G1 arrest.

As stated above, p27 is a critical regulator of cell cycle progression, inducing a strong cell cycle arrest when localised to the nucleus (Baldassarre 1999). To determine the effect exerted by ca-Akt on the function of p27, HEK293 cells were transfected with wild type p27 or with the corresponding T157A, T198A and T157A-T198A mutants, in the presence or absence of ca-Akt; subsequently, the relative efficiency of wild type or mutant p27 proteins to arrest proliferation in the presence of activated Akt was determined, by measuring the rate of BrdU incorporation. As shown in figure 13, the expression of wild type or mutant p27 proteins suppressed BrdU incorporation in all transfected cells (<1% of p27-positive cells incorporated BrdU). However, ca-Akt consistently relieved the block imposed by wild type p27, BrdU incorporation being augmented 11-fold in ca-Akt-transfected cells. Notably, the p27-T157A and T198A mutants were less sensitive to the mitogenic effects exerted by ca-Akt, and the double T157A-T198A mutant was even more resistant than each single mutants. In fact, BrdU incorporation of p27-T157A and T198A was increased of about 3.5-fold by ca-Akt whereas, BrdU incorporation of the double p27-T157A-T198A mutant was increased only of 2-fold. Therefore, Akt-mediated phosphorylation subverts p27-mediated growth arrest.

5. Akt-dependent phosphorylation modulates association of p27 with 14-3-3 proteins.

Akt-induced nuclear exclusion of p27 could be mediated by binding of phosphorylated p27 to 14-3-3 cytoplasmic scaffold proteins.

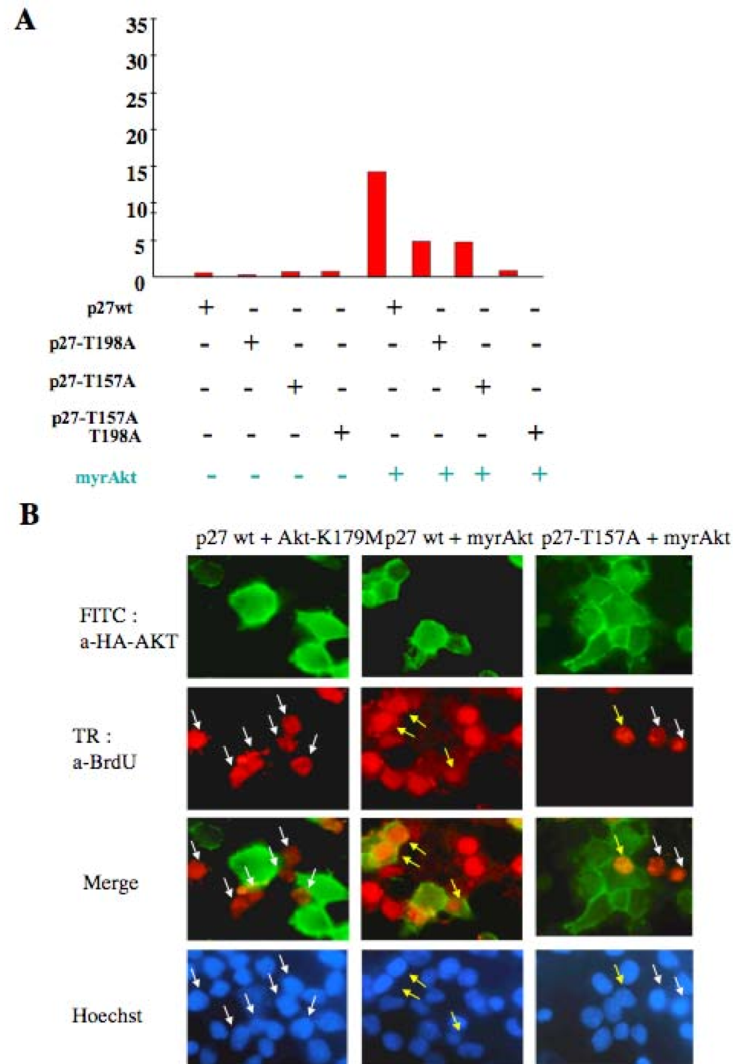


Fig. 13: ca-Akt rescues S-phase entry of p27-transfected cells. (A) BrdU incorporation in HEK293 cells co-transfected with FLAG-tagged wild type, T157A, T198A and T157A-T198A p27 (1μg) and HA-tagged myr-rAkt or Akt-K179M (2μg). After 36 h, cells were trypsinized, plated onto coverslips, pulsed with BrdU and processed for indirect immunofluorescence. Transfected cells (Fluorescein: green stain) were identified with a polyclonal anti-HA antibody whereas cells incorporating BrdU (Texas red: red stain) were identified with monoclonal anti-BrdU antibodies. Cell nuclei were counterstained with Hoechst (blue stain). The results are the average of 5 experiments in which at least 500 transfected cells were counted. Error bars indicate SD. (B) A representative experiment: yellow arrows indicate p27-transfected cells that, in the presence of myr-Akt, incorporate BrdU; white arrows indicate untrasfected cells that incorporate BrdU.

The 14-3-3s represent a group of conserved proteins that bind proteins phosphorylated on serine or threonine residues, leading to altered subcellular localization, possibly consequent to interference with nearby targeting sequences (Muslin et al. 2000). The motif optimal for association with 14-3-3 proteins overlaps with Akt consensus sequence (RS*X*pS*X*P and R*XXX*pS*X*P, respectively) (Datta et al. 1997; Diehl et al. 1998; Gesbert et al. 2000). In fact, it was shown that 14-3-3 proteins promote the cytoplasmic localization of Akt-phosphorylated FKHRL1, AFX (Kops et al. 1999) and BAD (Datta et al. 1997).

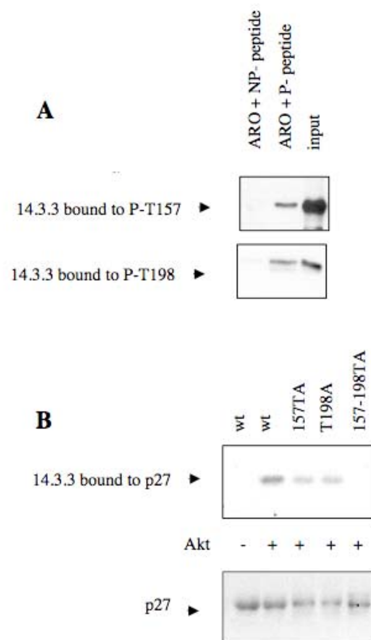


Fig. 14: Akt-dependent phosphorylation induces association of p27 with 14-3-3. (A) pull-down experiments with T157 and T198 peptides. Cell lysates (ARO cells) were following incubated with biotinylated non-phosphorylated T157 or T198 p27 peptides (*NP*) or phosphorylated T157 or T198 p27 peptides (*P*). After recovery with streptavidine beads, peptide-bound proteins were separated by SDS-PAGE and revealed with anti-14-3-3 antibodies. (B). Recombinant wild type or mutant p27 proteins were incubated with active Akt for 30 min at 30 °C, and subsequently separated by SDS-PAGE, and transferred to nitrocellulose membrane. Membrane was incubated with recombinant 14-3-3ε protein and revealed by anti-14.3.3 antibody.

We determined that Akt-phosphorylated p27 interacts with 14-3-3 proteins by peptide binding experiments using biotinylated non-phosphorylated and T157- and T198-phosphorylated peptides. The non-phosphorylated and phosphorylated peptides were incubated with lysates from different breast and thyroid cancer cells lines, and the peptide bound proteins were recovered with streptavidine-linked beads, separated by SDS-PAGE and identified by western blot with isoform-specific 14.3.3 antibodies (β, γ, ε, η, θ, ζ, τ). Both phosphorylated T157 and T198 residues were able to bind all 14.3.3 isoforms (though with different affinity), with binding depending on phosphorylation (Fig. 14A). In these "peptide pull-down" experiments the isoforms ε, θ, ζ were bound more strongly by both T157 and T198 containing peptides (not shown).

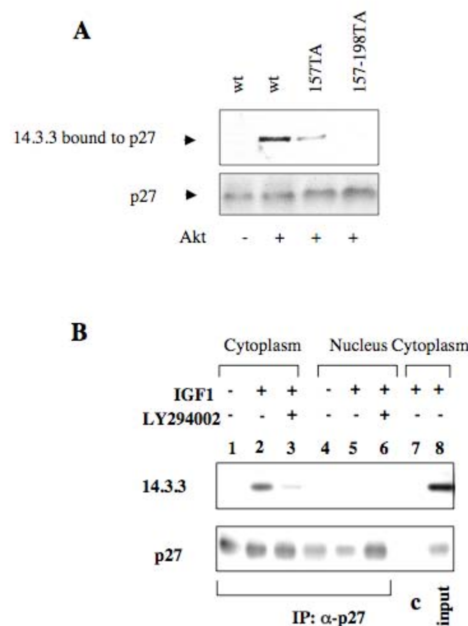


Fig. 15: Akt-dependent phosphorylation induces association of p27 with 14.3.3. (A) HEK293 cells were transfected with FLAG-p27, a V5-tagged 14.3.3ε with or without myrAkt. Proteins were immunoprecipitated with anti-FLAG and the immunocomplexes were immunoblotted with anti-V5. (B) MCF-7 cells were starved in 1% serum, pretreated with LY294002 or solvent alone and stimulated with IGF-1 for 15 min. Cells were lysed and cytoplasmic (C) and nuclear (N) proteins were prepared. 500 μg of proteins were immunoprecipitated with anti-p27 and analysed by Western blot with anti-14-3-3 or anti-p27. C: normal mouse serum.

In order to reconstitute the interaction between Akt-phosphorylated p27 and 14-3-3 *in vitro*, purified recombinant 6HIS-tagged, wild type and mutant p27 proteins were phosphorylated at 30 °C by ca-Akt, fractionated onto SDS-

PAGE and transferred onto a nitrocellulose membrane. Membranes were incubated with recombinant V5-tagged 14-3-3ε protein, and revealed with anti-V5 antibody. 14-3-3ε binds only Akt-phosphorylated but not unphosphorylated wild type p27, being the binding to the single T157A or T198A p27 mutants impaired of almost 50% and to T157A-T198A completely (Figure 14B). Moreover, in HEK293 cells transfected p27 complexes with 14-3-3 proteins in an Akt-dependent manner. The observation that replacing T157 and T198 with alanines abolishes binding completely, indicates that Akt-dependent phosphorylation of both sites is critical for 14.3.3 association *in vivo* (Fig. 15A). Finally, we determined that, at the endogenous levels of expression, association between p27 and 14-3-3 occurs in the cytosolic compartment, is stimulated by IGF-1 and inhibited by LY294002 (Fig. 15B). This series of results indicate that PI3K/Akt-mediated phosphorylation generates 14-3-3 binding sites on p27 thereby causing the assembly of 14-3-3-p27 complexes in the cytosolic compartment.

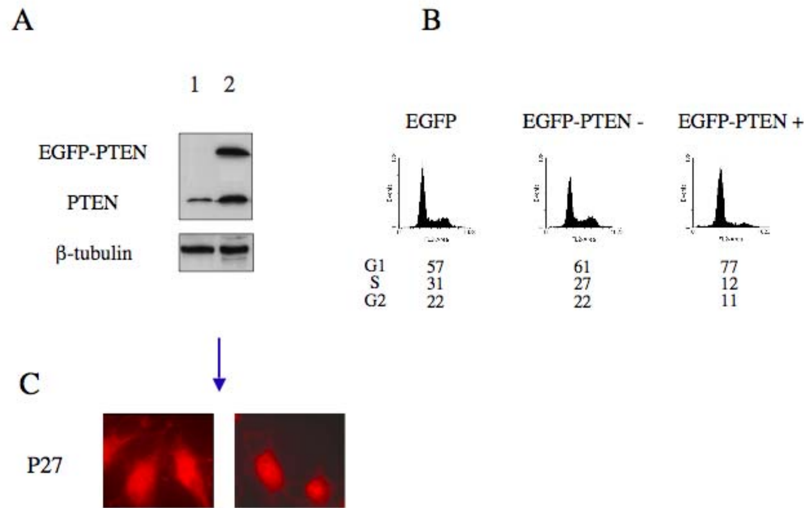
Although our results indicate that both T157 and T198 sites play a key role in Akt-dependent cytoplasmic redistribution of p27, it has been shown that the phosphorylation of p27 at T157 is already able to impair its nuclear import (Liang et al., 2002), thus providing an explanation for cytoplasmic localization of p27. Very recently, Sekimoto *et al.* (Sekimoto et al. 2004) reported that 14-3-3 proteins sequester the NLS of p27 from interacting with importin-α *in vitro*. This conclusion was further confirmed by the finding that T157 phosphorylation (much more than T198) is able to reduce the p27-importin-α association (Shin et al. 2005).

Therefore, our results suggest a novel mechanism whereby Akt promotes p27 redistribution and cell proliferation. This model implies direct phosphorylation of the NLS of $p27^{kip1}$, binding to 14.3.3, which in turn impairs binding to importin-α, and promotes p27 cytoplasmic relocalization. Activation of nuclear Cdk and cell cycle progression occur as a consequence of the exclusion of $p27^{kip1}$ from the nuclear compartment.

6. PI3K-dependent control of p27 localization in thyroid cancer cells.

To investigate the role of the PI3K/AKT pathway in the regulation of p27 function in the living cells, we selected a panel of representative thyroid cancer cells as cellular model systems. Cells were derived from PTC (TPC-1, NPA), FTC (WRO) and ATC (FRO, FB1) (Viglietto et al. 2002; Motti et al. 2004; Motti et al. 2005).

In this thesis we will focus on the role of the PI3K/Akt pathway on p27 phosphorylation and localization in thyroid cancer cells. A pathogenetic role of deregulated PI3K/Akt in thyroid tumorigenesis is suggested by the 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 (Eng et al. 1998). Akt activation occurs also in sporadic thyroid cancer through loss of PTEN (Bruni et al. 2000; Gimm et al.



2000), amplification of PI3KCA gene (Wu et al.) or increased protein Akt expression (Ringel

Fig. 16: PTEN regulates p27 localization. NPA cells were transfected with fused pEGFP-PTEN, harvested and green fluorescence emitted by EGFP was used to sort transfected from non-transfected cells. (A) Western blot analysis of transfected EGFP (1) or EGFP-PTEN (2) in NPA cells. (B) Cell cycle profile relative to cells transfected with pfEGFP and control pcDNA3 vector is shown in the left column (EGFP +); cell cycle profile relative to cells transfected with EGFP-PTEN, and sorted for being negative for EGFP is shown in the middle column (EGFP-PTEN-); cell cycle profile relative to cells transfected with pEGFP-PTEN, and sorted for being positive for EGFP is shown in the right column (EGFP-PTEN+). (C) Indirect immunofluorescence analysis of p27 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 was identified by Texas red-conjugated antibody.

2001, Vasko et al. 2004). The results presented here consistently point to a prominent role of PI3K/AKT signaling, and of p27 as a downstream effector of this pathway, in the process of thyroid carcinogenesis. These conclusions are in agreement with previous studies showing that sporadic thyroid tumors show PTEN mutation and/or deletion and that the PI3K/AKT pathway is required for growth of thyrocytes in response to insulin, IGF-1 and serum (Kimura et al. 2001; Zeng et al. 2000). The novel finding, here, is that increased signaling through the PI3K/Akt pathway in thyroid cancer cells causes cytoplasmic mislocalization of p27, through phosphorylation of T157 and T198.

The effects exerted by inhibition of PI3K/Akt pathway on cell proliferation and p27 localization in thyroid cancer cells were determined by flow cytometry experiments (Fig. 16). Overexpression of EGFP-PTEN resulted in a significant accumulation of NPA cells in the G1 phase of the cell cycle (Fig. 16B), and in parallel, PTEN induced accumulation of nuclear p27 as determined by indirect immunofluorescence experiments (Fig. 16C).
Similar results were obtained with pharmacological inhibitors of PI3K (LY294002, wortmannin) on the five representative cell lines TPC-1, NPA, WRO, FRO and FB-1 described above. The treatment of these cells with 20 μM LY294002 (or with 0.2 μM wortmannin) for 24 hours blocked proliferation inducing cells to accumulate in the G1 phase of the cell cycle, as

assessed by flow cytometry analysis (Table I), whch was reversed by antisense oligodeoxynucleotides that blocked p27 synthesis (not shown).

Table I
Flow cytometry analysis of thyroid cell lines.

Cells	Control	LY294002	Wortmannin
TPC-1			
G_0/G1	66.5	94.7	90.1
S	24.3	1.5	7
G2/M	9.0	3.8	2.9
NPA			
G_0/G1	52.9	81.8	78.8
S	31.9	15.1	14.2
G2/M	14.5	2.2	7
WRO			
G_0/G1	73	91	93
S	21	5	4
G2/M	6	4	3
ARO			
G_0/G1	69	85.3	84
S	24.6	7.6	11.8
G2/M	5.8	7.1	4.2
FB-1			
G_0/G1	51	67.2	74.2
S	23.4	5.1	10.1
G2/M	24.8	27	15.8

The most striking effect induced by LY294002 in thyroid cancer cells is a change in p27 subcellular localization (Figure 17A). Results were essentially similar for all cell lines, thus we present the data referring only to one (NPA). In proliferating control cells, p27 was detected almost exclusively in the cytoplasm, but upon PI3K block, the fraction of nuclear p27 markedly increased (ranging from 1:2 in TPC-1 to 1:100 cytoplasmic-to-nuclear ratio in FB-1). 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), indicating that such $p27^{kip1}$ accumulation was effective in inhibiting cell proliferation.

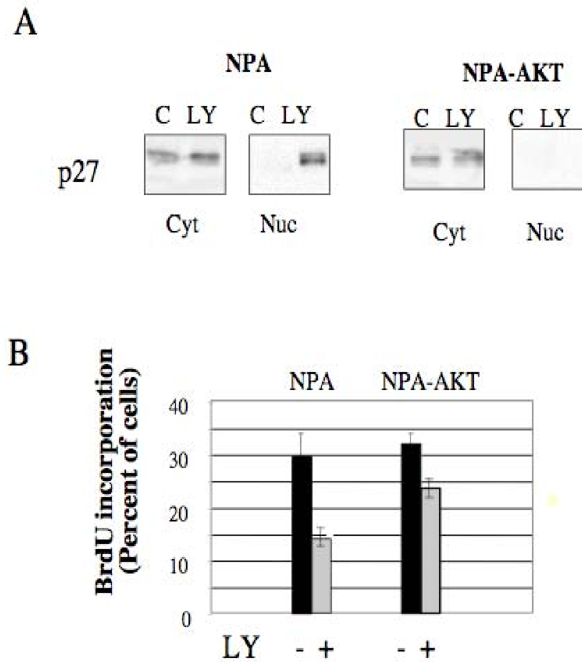


Fig. 17: AKT regulates p27 localization. (A). NPA and NPA-AKT cells were treated with DMSO or LY294002 (20 μM) for 24 hours, lysed and fractionated into cytoplasmic (cyt) and nuclear (nuc) fractions to determine p27 localization. Antibodies anti-SP1 and β-tubulin were used as controls. (B). BrdU incorporation of NPA and NPA-AKT cells treated with DMSO or LY294002 (20 μM) for 24 hours, incubated with 10 μM BrdU for 2 hours and processed for indirect immunofluorescence.

Subsequently, we investigated whether Akt mediated the effects exerted by the PI3K pathway on the subcellular localization of p27 in thyroid cancer cells. NPA cells were stably transfected with constitutively active ca-Akt, and clones that expressed elevated levels of active Akt were used for biological studies. Different resistant clones gave essentially similar results and thus we will refer to them as "NPA-Akt" cells. Treatment with LY294002 of NPA cells induced a marked accumulation of p27 in cell nuclei (Fig. 17A), whereas stable expression of activated Akt in NPA cells reduced LY294002-dependent p27 nuclear accumulation. In parallel with the effects exerted on p27 localization, NPA-Akt cells showed increased proliferative potential compared with NPA cells, as determined by BrdU incorporation assays (23.5% of NPA-Akt cells incorporated BrdU compared with 14% of NPA cells, respectively) (Fig. 17B).

Subsequently, we used phospho-specific antibodies to investigate whether the PI3K/Akt pathway regulates p27 localization in thyroid cancer cells through Akt dependent phosphorylation of T157 and T198. Using the anti-P-T157 and anti-P-T198 antibodies we found p27 phosphorylated at both these sites in proliferating NPA cells (Fig. 18A); exposure for 24 hours to LY294002 inhibited Akt and reduced phosphorylation of T157 and T198 (Figure 18A). Conversely, treatment with PI3K inhibitors (LY294002) had no effect on the phosphorylation status of S10, an additional amino acidic residue on $p27^{kip1}$ that has been suggested to phosphorylated by Akt (Fujita et al. 2002)

and to be critical for p27 cytoplasmic export (not shown). The finding that p27 phosphorylation of T157 and T198 was enhanced in NPA-Akt cells compared with NPA cells (Fig. 18A) formally demonstrated that in thyroid cancer cells these sites are phosphorylated in an Akt-dependent manner. As in the case of breast cancer (Viglietto et al. 2002; Motti et al. 2004), phosphorylation of endogenous $p27^{kip1}$ at T157 and T198 in NPA cells was restricted almost exclusively to the cytoplasmic compartment (Figure 19B).

A complementary approach to determine the relative importance of the different Akt-phosphorylated residues in the regulation of $p27^{kip1}$ subcellular

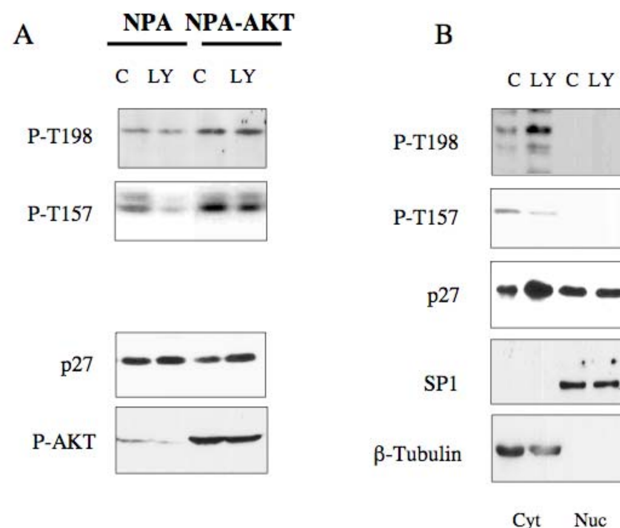


Fig. 17: Akt-phosphorylated p27 resides in the cytoplasmic compartment. (A). NPA and NPA-Akt cells were treated with DMSO or LY294002 (20 μM) for 24 hours and lysed. Total proteins (1 mg) were immunoprecipitated with a polyclonal anti-p27 antibody, separated onto a SDS-PAGE and immunoblotted with T157, and T198 phospho-specific and anti-p27 antibodies. Antibody to P-Akt S473 was used in immunoblots on lysates (40 μg) as control of the Akt activation status. (B). NPA-Akt cells were treated with DMSO or LY294002 (20 μM) for 24 hours and lysed. Cytoplasmic or nuclear proteins were immunoprecipitated with anti-p27 antibody, separated onto a SDS-PAGE and immunoblotted with T157 or T198 phospho-specific antibodies. Antibodies anti-SP1 and β-tubulin were used in immunoblots on lysates (40 μg) derived from cytoplasmic or nuclear proteins as controls of fractionation.

distribution was to transfect HA-tagged wild type and mutant p27 in thyroid cancer cells (NPA) and determine the effects exerted by pharmacological modulation of the PI3K pathway on p27 localization. NPA cells were plated

onto coverslips, transfected with wild type or mutant HA-tagged p27 and, after 24 hours, were treated with DMSO or LY294002 (20 μM) to suppress endogenous PI3K activity for additional 24 hours. P27 localization was determined by indirect immunofluorescence (Fig. 19). In NPA cells wild type p27 localized almost entirely to the cytoplasm (~83% of cells). However, treatment with LY294002 induced wild type p27 to localize to the nucleus (<20% of cells presented cytoplasmic staining). By contrast, HA-p27-T157A and HA-p27-T198A showed reduced cytoplasmic localization in absence of LY294002 (33% and 43% of cells showed cytoplasmic staining, respectively) but they still could be relocalized consistently to the nucleus in the presence of LY294002 (~20% and 18% of cells showed cytoplasmic staining, respectively); HA-p27-T157A-T198A showed almost complete nuclear localization (~17% and 11% of cells showing cytoplasmic staining in the absence and presence of LY294002, respectively). As expected from the phosphorylation data, the S10A mutant did not show any significant variation from the wild type HA-p27. These data demonstrate that both T157 and T198, but not S10 are implicated in PI3K-dependent cytosolic relocalization of p27.

7. In thyroid cancer activation of the PI3K-AKT pathway is associated with p27 cytosolic localization.

The ability of AKT to modulate cellular localization of p27 in established cell lines suggested that the presence of active AKT in thyroid tumors would correlate with expression of cytosolic p27. To this end, we analysed one hundred bioptic specimens derived from thyroid carcinomas for p27 expression and localization, and correlated them to the activity of the PI3K/Akt pathway. The status of the PI3K/Akt pathway was evaluated by determining the extent of Akt phosphorylation in primary thyroid carcinomas (10 FAs, 62 PTCs, 23 FTCs, and 5 ATCs) by immunoblot with the phospho-specific anti-S473 Akt antibody followed by film scanning and quantification. The status of Akt activity was confirmed at single cell level by immunostaining performed on all but one sample. Immunostaining analys completely overlapped immunoblot results. P-Akt was detected both in the nuclei and in the cytoplasm of tumor cells but was absent from stromal cells (see Fig. 20). Malignant tumors presented cytoplasmic P-Akt staining more often than benign tumors: 1/10 FAs, 27/62 PTCs and 12/23 FTCs, and 4/5 ATCs presented activated Akt (Fig. 20). The difference in the activation of Akt between adenomas (n=10) and carcinomas (n=90) was statistically significative when analysed with the 2-tailed Fisher's Exact Test (p value < 0.05).

Subsequently, samples were segregated into tumors with low pAkt staining (57/100) and high pAkt activity (43/100). We found no correlation between the activation status of AKT and p27 protein levels: low p27 expression was similarly found in tumors with low AKT and high AKT activity (see Motti et al. 2005). By contrast, we investigated the existence of a correlation between activated Akt and p27 localization by analyzing 33 samples of primary thyroid tumors (7 FAs, 18 conventional PTCs, 7 FTCs and 1 ATC) for p27 localization

by immunostaining. Benign tumors (6/7 adenomas) presented preferential nuclear p27 staining whereas malignant tumors (13/18 PTCs, 4/7 FTCs)

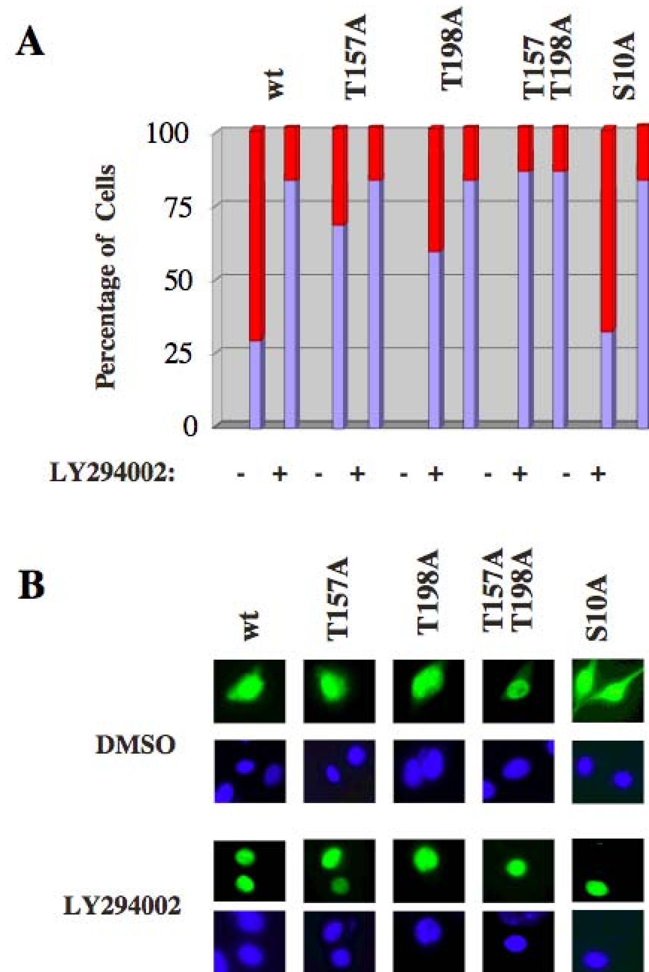


Fig. 19: T157 and T198 phosphorylation of p27 in thyroid cancer cells is critical for its localization. (A). HA-tagged wild type or mutant T157A, T198A and S10A p27 were transfected into NPA cells and treated with DMSO or LY294002. After 48 hours cells were fixed, permeabilized and processed for indirect immunofluorescence. Graph represents the median of 3 different experiments. Red bars, percentage of cells with cytoplasmic p27; Blue bars, percentage of cells with nuclear p27 (B). Representative immunofluorescence: p27 was revealed with FITC-conjugated secondary antibodies. Nuclei were identified by Hoechst staining. Magnification: 100X.

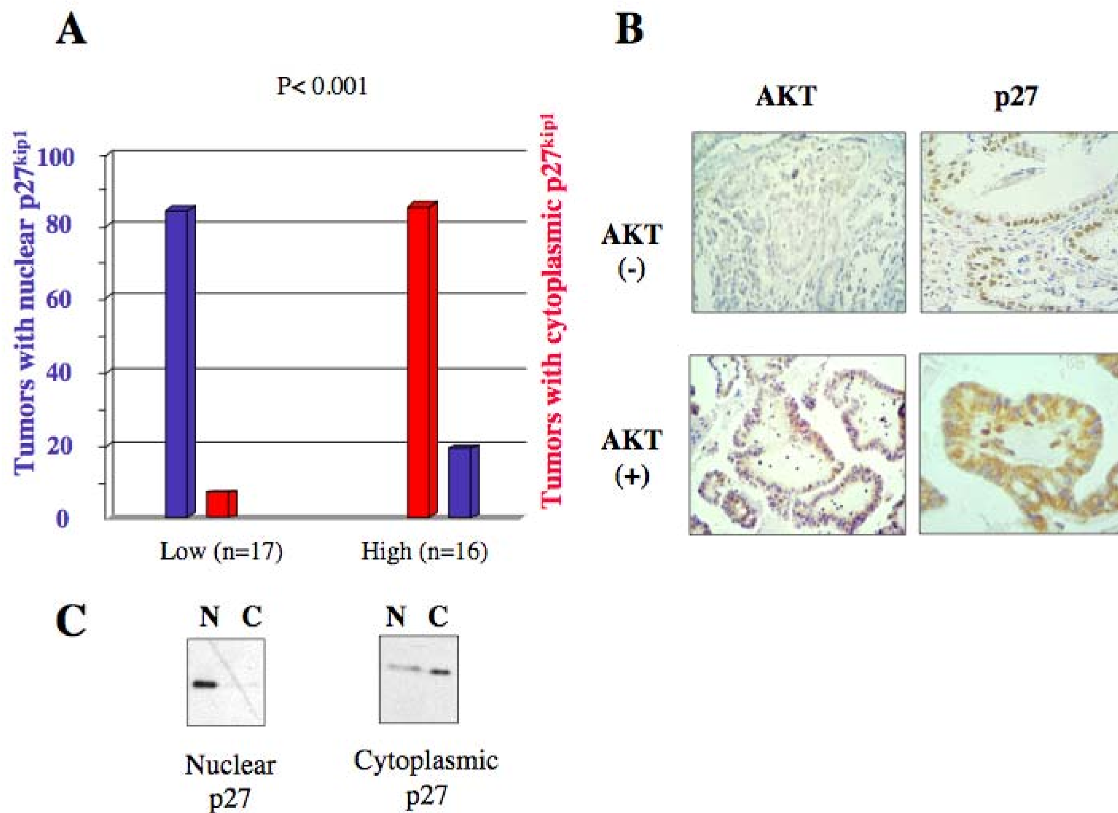


Fig. 20: Cytoplasmic mislocalization of p27 in primary thyroid cancer is associated with Akt activation. (A). Correlation between Akt activation and nuclear (blue bars) or cytoplasmic (red bars) p27 sub-cellular localization. Low: tumors with low Akt S473 phosphorylation; High: tumors with high Akt S473 phosphorylation. (B). Sections from 33 thyroid cancers were subjected to immunohistochemistry for S473 P-Akt and p27. Upper row: Papillary carcinoma negative for S473 P-Akt (left panel) and with the exclusively expression of p27 in cell nuclei (right panel). Lower row: S473 P-Akt positive papillary carcinoma (left panel) with expression of p27 in the cytoplasm of tumor cells (right panel). (C). Western blot analysis of p27 localization on nuclear- and cytoplasmic-enriched proteins.

presented consitent cytoplasmic p27 staining. When P-Akt levels were matched with p27 localization, a clear correlation was observed: p27 was detected exclusively in cell nuclei in 14/17 P-AKT negative specimens (82%) whereas, at the other end, p27 was detected in the cytosol of tumor cells in 16/18 (93%) of tumors with the highest level of P-AKT staining (P<0.002; χ^2 test with Yates correction) (see Table in Motti et al. 2005). A representative experiment is shown in Figure 20B. Subcellular localization of p27 was confirmed by immunoblot on cytoplasmic- and nuclear-enriched proteins prepared from 8 tumors with predominant p27 nuclear staining and 8 tumors with essentially cytoplasmic p27 staining (Figure 20C).

Finaly, we assessed T157 and T198 phosphorylation of p27 using anti-P-T157 or anti-P-T198 immunoblotting of p27 immunoprecipitates (see Table IV in Motti et al. 2005). Phosphorylation of p27 at T157 and T198 occurred in 6/7 tumors

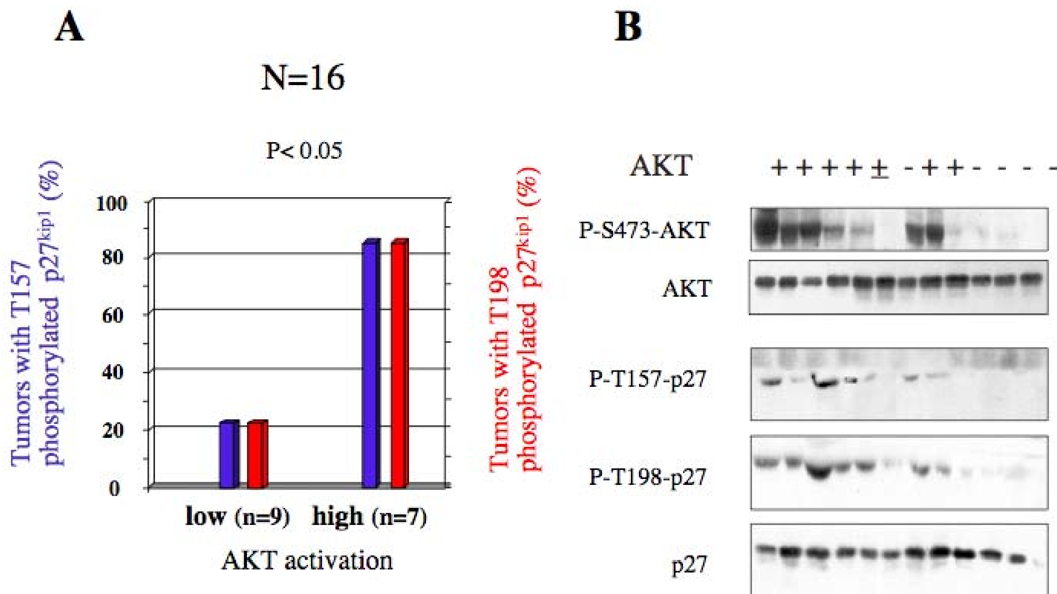


Fig. 21: Cytoplasmic mislocalization of p27 in primary thyroid cancer is associated with Akt activation (A). Correlation between Akt activation and nuclear (blue bars) or cytoplasmic (red bars) p27 sub-cellular localization. Low: tumors with low Akt S473 phosphorylation; High: tumors with high Akt S473 phosphorylation. (B). Sections from 33 thyroid cancers were subjected to immunohistochemistry for S473 P-Akt and p27. Upper row: Papillary carcinoma negative for S473 P-Akt (left panel) and with the exclusively expression of p27 in cell nuclei (right panel). Lower row: S473 P-Akt positive papillary carcinoma (left panel) with expression of p27 in the cytoplasm of tumor cells (right panel). (C). Western blot analysis of p27 localization on nuclear- and cytoplasmic-enriched proteins.

(3/3 FTCs, 2/3 PTCs, 1/1 ATCs) (85%) with activated Akt, but in only 2/9 tumors (1/3 FTCs, 1/6 PTCs) (25%) with unphosphorylated Akt (Figure 21A). In Figure 21B is shown a western blot with anti-phospho T157, T198, and total p27 antibodies. The correlation between AKT activation and T157/T198 phosphorylation of p27 was statistically significant (P<0.05; 2-tailed Fisher's Exact Test). All tumors that were positive for phosphorylated p27 presented

p27 in the cytoplasmic compartment, whereas the remaining cases, negative to the anti-phospho-antibodies, had nuclear staining.

Taken together these results demonstrate that activation of PI3K/Akt is observed in approximatively 40-50% in differentiated carcinomas (PTCs and FTCs, respectively) and in 80% of ATCs, thus contributing to development of thyroid carcinomas of all types. Experiments performed using five representative thyroid cancer cell lines have indicated that dysregulation of the PI3K/Akt pathway plays an important role in thyroid carcinogenesis and that p27 is a key target of the growth-promoting activity exerted by this pathway in thyroid cancer cells. According to our model (Fig. 22) Akt would regulate cell proliferation in thryoid cancer cells by inducing cytoplasmic sequestration of p27. By directly phosphorylating p27 at T157 and T198, Akt causes binding of p27 to 14.3.3 proteins and its retention of in the cytoplasm of cancer cells, precluding p27-induced G1 arrest.

An important issue to address is the function of the nuclear-to-cytoplasmic mislocalization of p27 in the development of thyroid tumors. Impaired import of p27 into cell nuclei has been proposed to facilitate cell proliferation as lowering p27 effective nuclear concentration under a critical threshold would remove inhibition from cyclin E-Cdk2 (Baldassarre et al. 1999). However, a broader analysis of the literature supports the idea that p27 may present some oncogenic cytoplasmic functions that foster carcinogenesis (Blagosklonny et al. 2002). This can explain the fact that in many thyroid tumors p27 is not simply lost but is also mislocalised. By analogy with the related Cdk inhibitor p21 (Asada et al. 1999), cytoplasmic p27 may suppress apoptosis or regulate migration, thus allowing cancer cells to dysregulate multiple cellular functions with one hit. Accordingly, a recent study has associated the presence of cytoplasmic p27 with increased migration in Akt expressing thyroid cancer cells (Vasko et al. 2004).

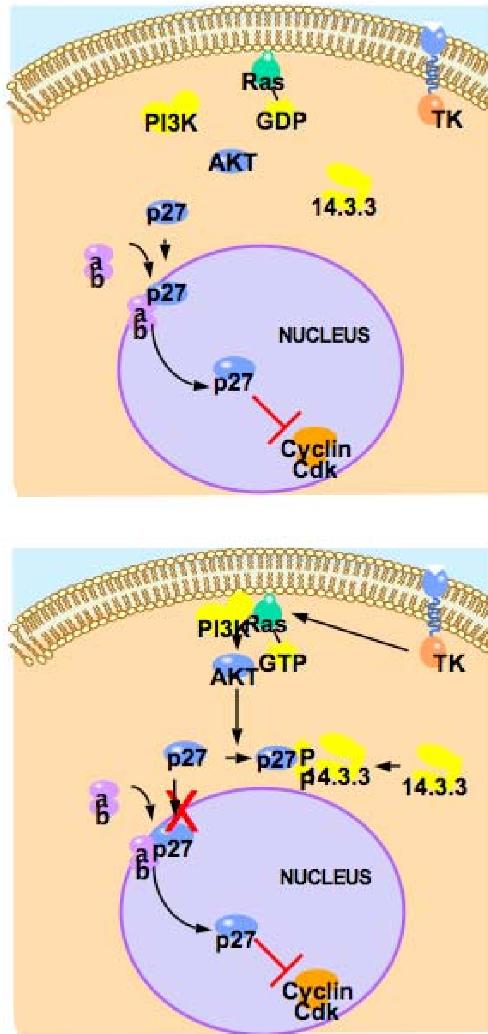


Fig. 22 Akt inhibits nuclear entry of p27 in thyroid cancer cells. (A) In normal epithelial cells, oncogenes are not activated thus Akt is inactive and p27 binds a-importin and is translocated to cell nucleus to inhibit cyclin E–cdk2. This prevents the activation of S phase–specific transcription factors, such as E2F, and cells become arrested in the G1 phase of the cell cycle. (B) In many thyroid carcinoma cells, the Akt pathway is overstimulated. Overstimulation can occur through oncogenic activation of tyrosine kinase receptors, such as RET/PTC, of G-protein such as RAS, or through inactivation of PTEN or activation of Akt itself. Activation allows Akt to phosphorylate p27 on two threonine residues induces binding to 14.3.3, impeding impotin-α-mediated nuclear entry of p27. Thus relegated to the cytoplasm, p27 is unable to control cdk2, which can remain active in the nucleus even in the presence of anti-mitogenic signals. Unrestricted cdk2 would activate E2F and allow unchecked cell proliferation. Therefore, barring p27 from the nucleus has hypothetically the same effect as loss of the protein.

Conclusions

The work performed in this thesis demonstrates that 27 phosphorylation by oncogenically activated Akt, a serine/threonine protein kinase that fosters cell proliferation and survival, disables the nuclear localization capacity of p27 in thyroid cancer cells. In fact, Akt can directly phosphorylate p27 on two residues, T157 and T198, both *in vitro* and *in vivo*. Akt-mediated phosphorylation causes the assembly of 14.3.3-p27 complexes in the cytosolic compartment and impairs its nuclear localization. Thus barred from the nucleus, p27 can no longer inhibit cell division, providing the tumor cell with consistent growth advantage. P27 is a direct inhibitor of Cdk2, one of the Cdks responsible for the activation of E2F1 transcription factors that promote DNA replication. Cdk2 is a nuclear protein, and cytoplasmic localization of p27 effectively separates it away from this target. This partition is precisely what Akt seems to be accomplishing in thyroid cancer cells.

In addition to defining a novel form of p27 regulation, our studies also suggest that p27 mislocalization is actively involved in the progression of thyroid cancer. Activated Akt is not detected in normal thyroid tissue, and the bulk of p27 is nuclear under these conditions. But the situation changes in cancer tissue. In fact, in approximately 40% of p27-positive primary thyroid carcinomas p27 resides in the cytoplasmic compartment: in tumors with low phosphorylation, p27 is strictly nuclear and Akt is inactive; conversely, in tumors with activated Akt, p27 is phosphorylated and mostly cytoplasmic.

Akt is a central component of the PI3K pathway, a pathway replete with oncogenically relevant components. The oncogenes RET/PTC, TRKA and RAS activate PI3K, and they both figure prominently as oncogenes in thyroid malignancies. On the other hand, PTEN, a major tumor-suppressor, functions to reverse the accumulation of Akt-activating phospholipids produced by PI3K. PTEN inactivation –either through inactivating mutations or loss of expression-occurs frequent in thyroid cancer. The consequence of these regulatory relationships is that Akt is commonly activated in many forms of thyroid cancer. The results deriving from these localization studies suggest that tumors that keep p27 in the cytoplasm may develop through pathways that are different from tumors that have lost p27 expression. Obviously, this implies that the genetic alterations involved in the dysregulation of p27 function in tumors could be distinct. In this sense, the effects exerted by the activation of RET/PTC or BRAF on p27 localization are expected to be different from those exerted by RAS mutation, PI3KCA mutation or PTEN loss. An important future objective of research will be the identification of the upstream genetic alterations that cause loss of p27 expression or mislocalization in thyroid tumors.

The finding that in patients affected by breast cancer, cytoplasmic p27 localization correlated with poor survival (Liang et al. 2002), suggest that, in a clinical perspective, both p27 location and levels could be used in predicting disease and therapy outcomes. On this basis, it will be interesting to determine

the survival rate of patients affected by thyroid carcinomas that show different levels of nuclear and cytoplasmic p27. Finally, the new data also raise some interesting possibilities for cancer therapies, at least in principle, since it should be feasible to restore the function of a misplaced p27 tumor suppressor protein. This has been shown in breast cancer cells where proper p27 localization has been restored either by pharmacological inhibition of the PI3K or by use of a neutralizing antibody elicited to the Erb2/HER2 receptor (Herceptin).
In conclusion, we are only beginning to understand the importance of the subcellular localization of p27 in the process of cell division and in the disregulation of the cell cycle observed in cancer and hence much more work is necessary to depict a complete picture.

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