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"Molecular mechanisms of cell cycle deregulation in thyroid cancer"

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“Molecular mechanisms of cell cycle deregulation in thyroid cancer”

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LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1. Motti M., **De Marco C**, Califano D, Fusco A, Viglietto G.. Akt-dependent T198 phosphorylation of cyclin-dependent kinase inhibitor p27kip1 in breast cancer. Cell Cycle 2004; 3(8): 1074-1080.

2. Motti M., Califano D, Troncone G, **De Marco C**, Migliaccio I, Palmieri E, Pezzullo L, Palombini L, Fusco A, Viglietto G. Complex regulation of the cyclin-dependent kinase inhibitor p27kip1 in thyroid cancer cells by the PI3K/AKT pathway: regulation of p27kip1 expression and localization. American Journal of Pathology 2005 Mar; 166(3): 737-749.

3. Chiappetta G, **De Marco C**, Quintiero A, Califano D, Gherardi S, Malanga D, Scrima M, Montero-Conde C, Cito L, Monaco M, Motti ML, Pasquinelli R, Agosti V, Robledo M, Fusco A, Viglietto G. Overexpression of the S-phase kinase-associated protein 2 in thyroid cancer. Endocr Relat Cancer. 2007 Jun; 14(2): 405-20.

4. Motti ML*, **De Marco C***, Califano D, De Gisi S, Troncone G, Persico A, Losito S, Fabiani F, Santoro M, Fusco A and Viglietto G. Loss of p27 expression through MAP kinase-dependent pathway in human thyroid carcinomas.
Cell Cycle 2007 Aug 13; 6(22) [Epub ahead of print]
*Maria Letizia Motti and Carmela De Marco equally contributed to this work.

ABSTRACT

Alteration of cell cycle is a hallmark of cancer. In particular, the importance of the G1/S transition in human tumour cells has been highlighted by the frequent observations of aberrant regulation of molecules involved in this process. As is the case with other common human carcinomas, a series of multiple alterations in cell cycle control-related gene products are involved in thyroid cancer pathogenesis.

The work done during my Doctorate thesis has contributed to a better understanding of the cell cycle control mechanisms involved in the development and progression of thyroid cancer.

The cyclin-dependent kinase inhibitor p27 is inactivated in thyroid cancer either through loss of expression or cytoplasmic mislocalization. In this study we report that mechanisms acting at the protein level are likely responsible for the downregulation of p27 expression observed in thyroid cancer.

The main mechanism regulating p27 abundance in tumour cell is the control of protein turnover, through the ubiquitine-proteasome pathway. Therefore, we have investigated the role of Skp2 in the development of human thyroid tumours and its relevance to p27 degradation. We have found that a significant fraction of thyroid carcinomas analysed presented high levels of Skp2 mRNA and protein, high p27 degradation activity and low p27 expression. Importantly, Skp2 overexpression was more frequently observed in anaplastic carcinomas than in differentiated tumours (83% ATC vs ≈ 41% PTC and FTC; $P<0.005$). As to the mechanism, we found that several thyroid anaplastic carcinomas presented gene amplification of locus encoding Skp2 at 5p13, as demonstrated by Q-PCR and FISH analysis. On the other hand, a significant fraction of thyroid carcinomas -both differentiated and anaplastic- overexpressed Skp2 mRNA and protein in absence of gene amplification. In this case, it is likely that the activation of genes such as *RET, RAS* and *BRAF* -that play a key role for the development of thyroid cancer- may be responsible for enhanced Skp2 expression observed in thyroid carcinomas. Accordingly, we found that the oncogenic version of these genes, *RET/PTC1, HRAS-V12* and *BRAF-V600E*, enhances Skp2 and decreases p27 expression through regulation of the MAP kinase-dependent pathway.

Finally, we investigated the oncogenic role exerted by Skp2 in thyroid cancer cells. Our results demonstrated that Skp2 is a critical determinant of thyroid cancer cell proliferation. In fact, suppression of Skp2 expression drastically reduced proliferation of thyroid cancer cells and, conversely, forced expression of Skp2 circumvented serum-dependency and contact inhibition in Skp2-negative cells by promoting p27 degradation.

These findings suggest that Skp2 may play an important role for the development of thyroid cancer and provide novel information on the

intracellular pathways that compromise the inhibitory function of p27 and, for this reason, are relevant for the comprehension of thyroid carcinogenensis.

BACKGROUND

1. HUMAN THYROID CANCER

Epithelial tumors derived from thyroid gland are usually classified into papillary carcinoma (PTC), follicular carcinoma (FTC) and anaplastic carcinoma (ATC) (Sherman 2003). 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 and Gharib, 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 (fig. 1). On the basis of clinical, histological and molecular observations, three distinct pathways are proposed for neoplastic proliferation of thyroid follicular cells, including hyper-functioning follicular thyroid adenoma (tumours that are almost always benign lesions without a propensity for progression), follicular thyroid carcinoma and papillary thyroid carcinoma.

Molecular alterations most relevant to thyroid tumorigenesis can be divided into four main categories: a) genetic alterations in signalling pathways**;** b) genetic alterations in differentiation factors; c) alterations of cell-cell and cell-matrix interactions; d) cell cycle deregulation.

1.1 Genetic alterations in signalling pathways

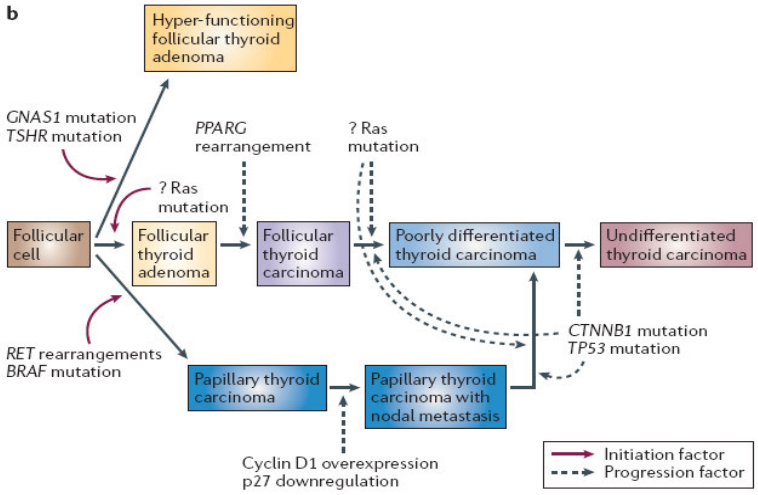
The principal cell-surface receptors that are involved in the regulation of thyroid follicular cell growth and function are shown in fig. 2. Alterations in key signaling effectors seem to be the hallmark of distinct forms of thyroid neoplasia.

Thyroid follicular cells express cell-surface receptors for thyroid-stimulating hormone (TSH), that induce cellular response mediated by cAMP.

Somatic mutations of the genes encoding TSH receptor or the associated Gsα subunit protein (gsp) determine their constitutive activation, resulting in increased intracellular cAMP levels and continuos stimulation of thyroid hormone synthesis and secretion.

Gain-of-function mutations of *TSHR* and *GSα* occur in hyper-functioning thyroid adenomas (Khron et al. 2005). By contrast, activating mutations of *TSHR* and *GNAS1* in thyroid malignancies are rare — this is consistent with

the clinical observation that hyper-functioning thyroid nodules are unlikely to be malignant (Matsuo et al. 1993, Spambalg et al. 1996).



Kondo T et al., Nat Rev Cancer 2006; 6(4):292-306.

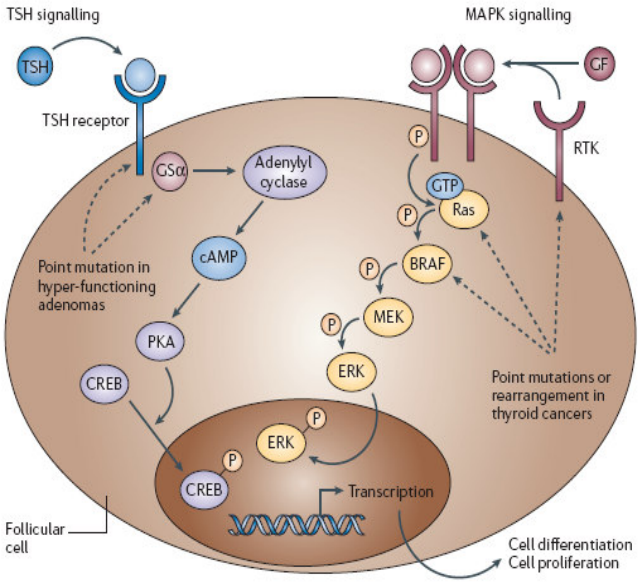
Figure 1. Model of multi-step carcinogenesis of thyroid neoplasm. Mutation of the *gsp* and *TSH* receptor genes are associated with benign hyperfunctioning thyroid nodules and adenomas. Genetic defects that result in activation of RET or BRAF represent early, frequent initiating events of cancer that can be associated with radiation exposure. Underexpression of the cyclin-dependent-kinase inhibitor $p27^{KIP1}$ and overexpression of cyclin D1 are strong predictors of lymph-node metastases in papillary thyroid carcinomas. Most poorly differentiated and undifferentiated thyroid carcinomas are considered to derive from pre-existing well-differentiated thyroid carcinoma through additional genetic events, including β-catenin (which is encoded by CTNNB1) nuclear accumulation and p53 inactivation, but de novo occurrence might also occur.

RET was the first activated receptor-tyrosine kinase to be identified in thyroid cancer. It activates several signalling pathways, including extracellular regulated kinase (ERK, also known as MAPK kinase 1 and 3), phosphatidylinositol 3-kinase (PI3K), MAPK p38 and C-JUN kinase (JNK, also known as MAPK kinase 8).

Gain-of-function mutations of *RET* are involved in sporadic and familial C-cell-derived medullary thyroid carcinoma, including multiple endocrine neoplasia 2A (MEN2A), MEN2B and familial medullary thyroid carcinoma (Marx et al. 2005).

By contrast, chimeric oncogenes, designated *RET*/PTC, are implicated in the development of papillary carcinoma (Tallini et al. 2001). Although wild-type *RET* is not normally expressed in follicular cells, *RET/PTC* chimeric oncoproteins that lack a signal peptide and transmembrane domain are expressed in the cytoplasm of follicular cells, under the control of the newly acquired promoters. Ligand-independent tyrosine phosphorylation is induced by constitutive dimerization of the fusion proteins.

The high incidence of *RET* rearrangements in childhood papillary carcinomas following the Chernobyl accident indicates a role for radiation damage in the genesis of these paracentric inversions (Nikiforov et al. 1997, Smida et al. 1999).



Kondo T et al., Nat Rev Cancer 2006; 6(4):292-306.

Figure 2. Cell signaling pathways in follicular cell. TSH activates this receptor and G proteins such as GSα at the cell surface of follicular cells, and induces intracellular production of cyclic AMP (cAMP) by adenylyl cyclase. cAMP stimulates the cAMPdependent protein kinase A (PKA), which in turn phosphorylates cytoplasmic and nuclear target proteins. One PKA substrate is the nuclear transcription factor CREB, which activates the transcription of cAMP-responsive genes after being phosphorylated by PKA. Growth factors (GF) induce receptor tyrosine kinase (RTK) dimerization, resulting in phosphorylation of specific tyrosine residues within the cytoplasmic tail. In cooperation with receptor adaptors, phosphorylated RTK activates Ras by catalyzing the replacement of GDP with GTP. In its GTP-bound form, Ras activates the kinase activity of BRAF and its downstream signalling cascade. BRAF phosphorylates the mitogen-activated protein kinase (MAPK) kinase (MEK), which phosphorylates and activates extracellular-signalregulated kinase (ERK). Activated ERK migrates to the nucleus where it phosphorylates and activates various transcription factors that are involved in cell proliferation and differentiation, such as MYC and ELK1.

In cooperation with receptor adaptors, phosphorylated RTK activates Ras by catalysing the replacement of GDP with GTP. There are three different Ras genes, H-Ras, K-Ras and N-Ras.

Their function is to convey signals originating from tyrosine kinase membrane receptors to a cascade of mitogen-activated phosphokinases (MAPK) which activate the transcription of target gene resulting in cell proliferation. Oncogenic Ras activation is due to point mutations affecting the GTP-binding domain (codons 12 or 13) in exon 1 or the GTPase domain (codon 61) in Exon 2 which lock the protein in the active GTP-bound form.

Unlike other solid neoplasms, Ras is the least prominent participant in thyroid carcinogenesis. Ras mutations are more common in lesions with follicular architecture (including follicular carcinoma and follicular variant papillary thyroid carcinoma) than in typical papillary thyroid carcinoma (Zhu et al. 2003).

Some have argued that the presence of Ras mutations in benign tumours implicates this as an early event in thyroid tumorigenesis (Namba et al.1990).

BRAF encodes a serine/threonine kinase that transduces regulatory signals through the Ras–Raf–MEK–ERK cascade. Gain-of-function *BRAF* mutation provides an alternative route for the aberrant activation of ERK signalling Among point mutations of exon 15 in thyroid cancers, BRAFV600E is the most common alteration in sporadic papillary carcinoma (Xing 2005). *BRAF* mutations are found in 29–69% of papillary thyroid carcinoma but not in follicular thyroid carcinoma, and in up to 13% of poorly differentiated thyroid carcinoma and 35% of undifferentiated thyroid carcinoma (Xing 2005, Davies et al. 2002, Namba et al. 2003, Nikiforova et al. 2003, Soares et al. 2004). *BRAF* mutations in papillary thyroid carcinoma correlate with distant metastasis and more advanced clinical stage (Namba et al. 2003), and occur at a significantly higher frequency in older patients; a gender difference is still controversial (Sobrinho-Simoes et al. 2005, Trovisco et al. 2005, Nikiforova et al. 2003, Soares et al. 2004).

1.2 Genetic alterations in differentiation factors

The peroxisome proliferator-activated receptor-γ (PPARγ) is the subject of a unique and interesting rearrangement in follicular thyroid carcinoma. PPARγ, which is encoded by *PPARG* (located on chromosome 3p25), is a member of the steroid nuclear-hormonereceptor superfamily that forms heterodimers with retinoid X receptor. It is best known for its differentiating effects on adipocytes and insulin-mediated metabolic functions (Desvergne and Wahli 1999). Paired-box gene 8 (*PAX8*)–*PPARG* rearrangements were first identified in thyroid neoplasms with a cytogenetically detectable translocation t(2;3)(q13;p25) that generates a chimeric gene encoding the DNA-binding domain of the thyroid-specific transcription factor PAX8 and domains A–F of PPARγ (Kroll et al. 2000). The *PPARG* rearrangement functions through a dominant- negative effect on the transcriptional activity of wild-type PPARγ. The fusion oncoprotein contributes to malignant transformation by targeting several

cellular pathways, some of which are normally engaged by PPARγ (Gregory Powell et al. 2004).
The *PPARG* rearrangement seems to be almost restricted to follicular thyroid adenoma (0–31%) and follicular thyroid carcinoma (25–63%), with the initial indication that it correlates with a vasculo-invasive phenotype (Nikiforova et al. 2003, Kroll et al. 2000, Cheung et al. 2003).

1.3 Alterations of cell-cell and cell-matrix interactions

Tumour cells are held together by direct cell–cell contact and by adhesion to the extracellular matrix (fig. 3). Loss of adhesion is thought to promote tumour invasiveness and increase the metastatic potential of many human carcinomas.
Cadherins belong to a family of single-transmembrane calcium-dependent cell–cell adhesion proteins. There are three classical cadherins: neuronal (N)-, placental (P)- and epithelial (E)-cadherin. E-cadherin (which is encoded by *CDH1* on chromosome 16q22) is highly expressed in normal thyroid and benign adenomas. Expression of E-cadherin is maintained in some well-differentiated thyroid carcinomas, including minimally invasive lesions; however, in widely invasive, recurrent or metastatic thyroid carcinomas expression of E-cadherin is low or absent (Brabant et al. 1993, Scheumman et al. 1995). Expression of E-cadherin in undifferentiated thyroid carcinoma is extremely low. This is not likely to be attributable to mutation as somatic mutations of *CDH1* are infrequent in thyroid cancers (Soares et al. 1997). Methylation of the *CDH1* promoter might underlie underexpression (Kato et al. 2002).

β-Catenin (which is encoded by *CTNNB1* on chromosome 3p22–21.3) binds the cytoplasmic tail of cadherins, and has two main functions in cell regulation — as a cadherin-mediated adhesion regulator and as a mediator of Wnt–β-catenin signalling, which targets cyclin D1 and MYC. Normal thyroid follicular cells express membranous β-catenin, expression of which is progressively reduced with loss of tumour differentiation (Cerrato et al. 1998). Although increased cytoplasmic β-catenin is observed in thyroid cancer cells, it seems that nuclear localization is restricted to poorly differentiated (21%) and undifferentiated thyroid carcinomas (48%) (Garcia-Rostan et al. 2001). Nuclear localization of β-catenin is induced by *CTNNB1* mutations rather than *APC* mutation in thyroid cancers — mutations of *CTNNB1* are observed in 0–25% of poorly differentiated thyroid carcinomas and up to 66% of undifferentiated thyroid carcinomas, but not in well-differentiated thyroid carcinomas. These observations indicate that *CTNNB1* mutation contributes to progression towards poorly differentiated or undifferentiated thyroid carcinomas.
Taken together, thyroid cancer represents a model of solid neoplasia in which the loss of adhesion serves to promote tumour invasiveness and increase metastatic potential.

1.4 Cell cycle deregulation

The growth activity of well-differentiated thyroid carcinoma is low compared with poorly differentiated and undifferentiated thyroid carcinomas; the MIB-1 index is 1–3% in well-differentiated thyroid carcinomas, 6–7% in poorly differentiated thyroid carcinomas and 14–52% in undifferentiated thyroid carcinomas (Katoh et al. 1995, Yoshida et al. 1999, Kjellman et.al 2003). Altered cell-cycle regulators govern these differences in growth activity. Progression factors (for example, cyclin D1, cyclin E1, cyclin-dependent kinases (CDKs) and E2Fs) and competitor factors (for example, retinoblastoma protein (RB), $p16^{INK4A}$, $p21^{CIP1}$, $p27^{KIP1}$ and p53) regulate the transition from G1 to S phase (fig. 3).

Cyclin–CDK complexes promote cell-cycle progression through inactivation of RB. Although nuclear immunoreactivity for cyclin D1 (which is encoded by *CCND1* on chromosome 11q13) and cyclin E1 (which is encoded by *CCNE1* on chromosome 19q12) are not detectable in normal thyroid follicular cells, expression of cyclin D1 and cyclin E1 is observed in approximately 30% and 76% of papillary thyroid carcinomas, respectively (Basolo et al. 2000, Lazzereschi et al. 1998, Brzezinski et al. 2004).

Furthermore, cyclin D1 overexpression correlates with metastatic spread in papillary thyroid carcinomas, and significant overexpression of cyclin D1 is observed in undifferentiated thyroid carcinoma (Wang et al. 2000). The copy number of *CCND1* is amplified in a number of human neoplasms; however, neither major genetic alterations nor amplification of *CCND1* and *CCNE1* have been found in thyroid cancers (Lazzareschi et al. 1998). So, overexpression of cyclins is probably a secondary effect that is induced by other genetic aberrations in thyroid tumours.

CDK inhibitors are commonly downregulated in thyroid neoplasia. There is progressive loss of $p21^{CIP1}$ (which is encoded by *CDKN1A* on chromosome 6p21 with advancing tumour stage of papillary thyroid carcinomas, and 13% of papillary thyroid carcinomas harbor *CDKN1A* deletions (Brzezinski et al. 2005).

Normal and hyperplastic follicular cells show strong immunoreactivity for p27KIP1 (which is encoded by *CDKN1B* on chromosome 12p13), whereas p27KIP1 expression is significantly reduced in papillary thyroid carcinoma, mainly metastatic forms and undifferentiated thyroid carcinoma (Resnick et al. 1998, Baldassarre et al. 1999). 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. 2004). Importantly, $p27^{kip1}$ degradation is enhanced in many aggressive human tumors. The frequency with which this is observed suggest that loss of $p27^{kip1}$ may confer a growth advantage to these cancers.

Nonetheless, certain carcinomas of the thyroid contain normal or even increased levels of $p27^{kip1}$ but, strangely, the protein has shifted location in these cancers (Baldassarre et al. 1999). Normally $p27^{kip1}$ resides in the nucleus, but in these tumors it can be found instead in the cytoplasm.

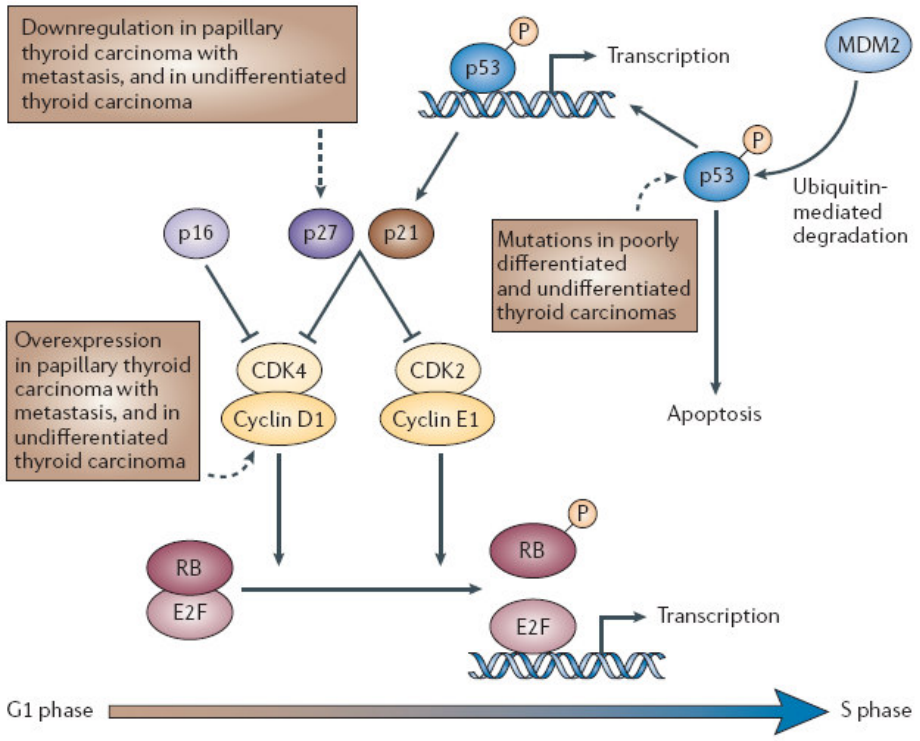


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Figure 3. Cell-cycle regulation in follicular cells. Cyclin D1 and cyclin E1 cooperate to control the G1 to S phase transition through interactions with retinoblastoma protein (RB). Cyclin D1 and cyclin E1 heterodimerize with cyclin-dependent kinases (CDKs) 4 and 2, respectively, to inactivate the tumour suppressor RB by phosphorylation. Active RB functions as a repressor of E2F transcription factors, whereas inactivation (phosphorylation) of RB allows E2F transcriptional activity. E2F activates the transcription of genes that are involved in the G1 to S phase transition, such as DNA polymerase and thymidine kinase. The CDK inhibitors $p16^{INK4A}$, $p21^{CIP1}$ and $p27^{KIP1}$ impair the activity of cyclin CDK complexes, thereby preventing phosphorylation of RB. The CDK inhibitors therefore function as tumour suppressors. The tumour suppressor p53 induces cell-cycle arrest by upregulating $p21^{CIP1}$, which initiates apoptosis. The function of p53 is controlled by negative regulators, including MDM2. The MDM2 protein targets p53 for ubiquitinmediated degradation, constituting a feedback loop to maintain a low concentration of p53 in the cells.

2. THE CYCLIN-DEPENDENT KINASE INHIBITOR, P27, AND THE PATHWAYS THAT AFFECT ITS EXPRESSION IN CANCER

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 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 Reed 2000; Sherr and Roberts 1999; Vidal and Koff 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 and Pledger 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 and Reed 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. 4).

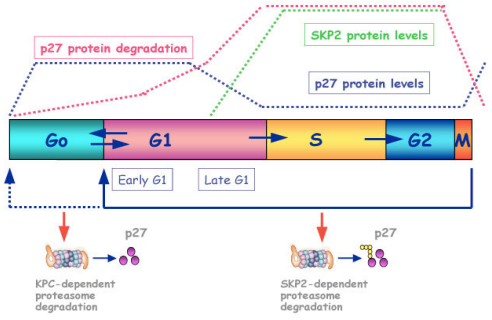


Figure 4. Regulation of p27 expression during cell cycle. In early and mid G1 p27 degradation is dependent on KPC whereas in late G1 and S phase its degradation is SKP2 dependent.

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.5). 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.

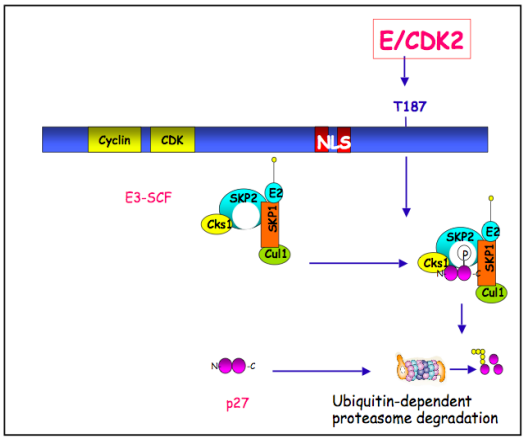


Figure 5. Molecular mechanims of p27 degradation. Degradation of p27 through the ubiquitin-proteasome pathway is a three-step process that requires: (i) phosphorylation of p27 at threonine 187 by cyclin E/Cdk2; (ii) recognition of T187-phosphorylated p27 by the ubiquitin ligase; (iii) SCF^{Skp2}-dependent ubiquitination and degradation of T187-phosphorylated p27.

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.

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. 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 (Rho A and Ral A) may also be required (Hu et al. 1999; Yamazaki et al. 2001). How oncogenic Ras induces down-regulation 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 and Hunter 2001). Converging data from the recent literature indicate that both PI3K and MAP kinase pathways are involved in the regulation of p27 expression.

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).

Different molecular mechanisms account for the MAP kinase-dependent decrease in the intracellular abundance of p27 including decreased protein p27 synthesis, decreased p27 protein stability or increased Cdk2 activity (Rivard et al. 1999). Furthermore, Ras signalling induces expression of SKP2, involved in p27 degradation, through the binding of GA-binding protein, an Ets-family transcription factor, to the SKP2 promoter (Imaki et al. 2003).

3. UBIQUITIN LIGASES IN CELL CYCLE CONTROL

The intracellular levels of p27 as well as of other CKIs vary periodically during the different phases of the cell cycle: they are generally high when cells are in G1 and very low when cells enter into S phase (Evans et al. 1983). This is the result of a constant synthetic rate coupled with a defined window in the cycle of specific proteolysis, which is executed by the ubiquitin-proteasome system (UPS) (Glotzer et al. 1991). The expression of cyclins during cell division is also periodic since most of them are targeted for degradation by the UPS.
Therefore, the cell cycle is predominantly regulated by two types of post-translational protein modification — phosphorylation and ubiquitylation (fig. 6).

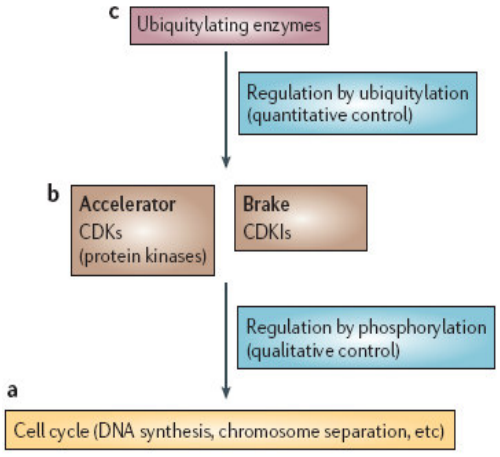
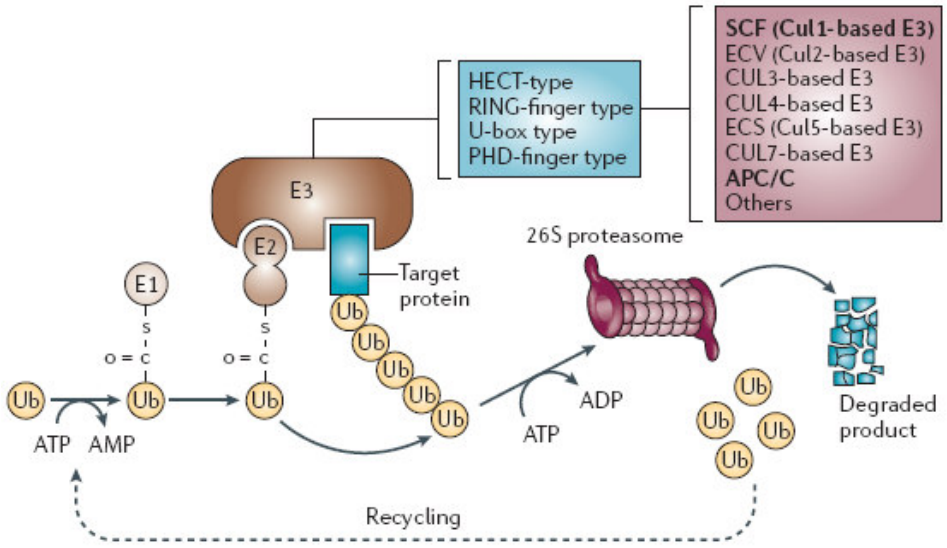


Figure 6. Three-layer regulation cell cycle. The immediate phenomena of the cell cycle, including DNA synthesis and chromosome separation (layer a), are qualitatively controlled by phosphorylation. Movement through the cycle (layer b) depends on the activity of cyclin-dependent kinases (CDKs), which are promoted by accelerators — cyclins — and antagonized by brakes — CDK inhibitors (CKIs). The protein levels of cyclins, CKIs and many other cell-cycle-related regulators are quantitatively controlled by ubiquitylating enzymes (layer c). Accumulating clinical evidence shows various alterations in the ubiquitylation of cell-cycle regulators in the aetiology of many human malignancies.

Nakayama KI and Nakayama K, Nat. Rev. Cancer 2006; 6(5):369-81.

The UPS comprises two discrete steps: the covalent attachment of multiple ubiquitin molecules to the protein substrate and degradation of the polyubiquitylated protein by the 26S proteasome complex (Hershko et al. 1983) (fig. 7).



Nakayama KI and Nakayama K, Nat. Rev. Cancer 2006; 6(5):369-81.

Figure 7. Overview of the ubiquitin-proteasome pathway. Ubiquitin (Ub) is a small 8-kDa protein, which is first transferred to the ubiquitin-activating enzyme, E1, in an ATPdependent manner. This activated ubiquitin is then transferred to the ubiquitin conjugating enzyme, E2. Finally, the ubiquitin is covalently attached to the target protein by E3 ubiquitin ligase, leading to the formation of a polyubiquitin chain. The polyubiquitylated protein is recognized by the 26S proteasome, and is destroyed in an ATP-dependent manner. There are many E3 ubiquitin ligases, which are categorized into four major classes: HECT-type, RING-finger-type, U-box-type and PHD-finger-type. RING-finger-type E3s are further divided into subfamilies, including cullin-based E3s, which constitute one of the largest classes of E3s. There are seven cullin-based E3s including the SKP1–CUL1–F-box-protein (SCF) complex and the anaphase-promoting complex/cyclosome (APC/C).

The first step is mediated by at least three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). An involvement of E1 in cancer has not been described, and only a few reports have linked E2 to cancer development (Okamoto et al. 2003).
By contrast, a large amount of evidence indicates that deregulation of E3 ligase, which is involved in many biological systems, often results in cancer development. E3 components in the UPS are thought to be primarily responsible for the specific recognition of a large number of target proteins (Hershko et al. 1983). This requires both specificity and versatility, which are provided by the existence of 500–1,000 different E3 ligases. These are now

categorized into four major classes on the basis of their specific structural motif: HECT-type, RING finger- type, U-box-type or PHD-finger-type (fig. 7). RING-finger-type E3s are thought to be the largest family and are further divided into subfamilies; one of these, the cullin-based E3 subfamily, is one of the largest single classes of E3. There are seven cullin-based E3s, including the SKP1-CUL1–F-box-protein (SCF) complex (Nakayama KI and Nakayama K 2005) and the anaphase-promoting complex/ cyclosome (APC/C) (Harper et al. 2002, Castro et al. 2005), both of which are involved in the proteolysis of core components of the cell-cycle machinery. The SCF complex consists of three invariable components — RBX1 (RING-finger protein), CUL1 (scaffold protein), and SKP1 (adaptor protein) — as well as one variable component, known as an F-box protein, that binds through its F box motif to SKP1 and is responsible for substrate recognition.

Three F-box proteins — SKP2 (FBXL1), FBW7 (FBXW7) and β-transducin repeat-containing protein (β-TRCP) (FBXW1/11) — are thought to be involved in cell cycle control (the functions of most other F-box proteins remain unknown). The APC/C is structurally similar to the SCF complex, and consists of invariable core components — APC11 (RBX1-related RING-finger protein), APC2 (CUL1-related scaffold protein) and at least 11 other components without a defined role — as well as a variable component known as an activator. There are two such variable components in mitotically cycling cells — cell division cycle 20 (CDC20) and CDH1 (also known as HCT1) — and they confer substrate specificity in the same way that F-box proteins do in the SCF complex (Harper et al. 2002, Castro et al. 2005). Additional APC/C activators function during meiosis and in non-dividing cells.

Despite the structural and biochemical similarities between the SCF complex and the APC/C, their cellular functions are different. This is highlighted by the timing of action of each E3 complex: the APC/C is active from mid-mitosis (anaphase) to the end of G1 phase, whereas the SCF complex, although originally thought to function mainly at the G1–S transition, is active from late G1 to early M phase.

3.1 The SCF^{SKP2} complex

The system that targets p27 for degradation is represented by the SCF^{SKP2} complex (Carrano et al. 1999), where SKP2 protein is the variable F-box protein component, responsible for substrate recognition.

SKP2 was originally discovered as a protein that associates with cyclinA–CDK2 in transformed cells (Zhang et al. 1995), and is now known to be an F-box protein of the SCF complex. In conjunction with SKP2, the SCF complex targets p27 (Carrano et al. 1999, Sutterluty et al.1999), p21 (REFS Yu et al. 1998) and p57 (Kamura et al. 2003) CKIs for degradation (fig. 4). To date, it

has also been reported that SKP2 targets p130, cyclin A, cyclin D1, free cyclin E, E2F1, ORC1, CDT1, CDK9, MYC, B-MYB, SMAD4, RAG2, UBP43, FOXO1 and papillomavirus E7 (Nakayama KI and Nakayama K 2005).
However, it is p27 that seems to be the primary target of SKP2, given that *Skp2–/–* mice show a marked accumulation of p27 (Nakayama et al. 2000), and that prominent cellular phenotypes apparent in *Skp2–/–* mice— including nuclear enlargement, polyploidy and an increased number of centrosomes that are probably caused by overreplication of chromosomes and centrosomes — disappear in *Skp2–/– p27–/–* double-mutant mice (Nakayama et al. 2004). It has been widely accepted that p27 is a tumour suppressor, not only because of its activity as a CKI, but also because of evidence from mouse models (Fero et al. 1996, Kiyokawa et al. 1996) and the marked correlation between reduced p27 levels and poor prognosis found in clinical studies of patients with cancer (Bloom and Pagano 2003). Indeed, a reduction in the concentration of p27 is common in many types of human malignancies. However, in contrast with other tumour suppressors such as p53 or RB, mutation or deletion of the *p27* gene is an uncommon event in the development of human cancers, indicating that deregulation of p27 expression in human tumours is often due to post-transcriptional mechanisms.
It has become evident that SKP2 expression is inversely correlated with levels of p27 in many cancers, and also with the grade of malignancy in certain human tumours (table 1).

Cancer type	Observed alterations
SKP2 (SCF)	
Oro-pharyngo-laryngeal cancer	Correlation with poor prognosis; inverse correlation with p27 expression
Oesophageal cancer	Correlation with poor prognosis; inverse correlation with p27 expression
Gastric cancer	Correlation with poor prognosis; inverse correlation with p27 expression
Colon cancer	Correlation with poor prognosis; inverse correlation with p27 expression
Biliary tract cancer	Correlation with poor prognosis; no correlation with p27 expression
Lung cancer	Correlation with poor prognosis; inverse correlation with p27 expression; gene amplification; no correlation with p27 expression
Melanoma	Correlation with poor prognosis; inverse correlation with p27 expression
Glioma/Glioblastoma	Correlation with poor prognosis; inverse correlation with p27 expression; gene amplification
Breast cancer	Correlation with poor prognosis; inverse correlation with p27 expression
Renal cell cancer	Correlation with poor prognosis
Prostate cancer	Correlation with prognosis; inverse correlation with p27 expression
Transitional cell cancer	Correlation with poor prognosis
Cervix cancer	Correlation with poor prognosis; no correlation with p27 expression
Endometrial cancer	Correlation with poor prognosis
Ovarian cancer	Correlation with poor prognosis
Kaposi sarcoma	Correlation with poor prognosis; no correlation with p27 expression
Soft Tissue sarcoma	Correlation with poor prognosis; no correlation with p27 expression
Lymphoma and leukaemia	Correlation with poor prognosis; inverse expression of p27; no correlation with p27 expression

Table 1: SKP2 alterations in human malignancies.

In addition, frequent amplification and overexpression of the *SKP2* gene has been observed in lung cancers (Yokoi et al. 2002, Yokoi et al. 2004) and in cell lines expressing high-risk human papilloma virus (Dowen et al. 2003). Other components of the ubiquitylation machinery for p27, primarily CKS1, have oncogenic potential in patients with colorectal carcinoma (Shapira et al. 2004), and overexpression of SKP2 or CKS1 is strongly and independently associated with a loss of tumour differentiation and poor survival (Shapira et al. 2005). The oncogenic potential of SKP2 has also been shown in transgenic mouse models (Shim et al. 2003).

AIMS OF THE STUDY

Inactivation of cyclin-dependent kinase inhibitor p27 plays an important role in human cancer pathogenesis. In thyroid cancer p27 reduction can be ascribed to both cytoplasmic sequestration and loss of expression triggered by post-trascriptional modifications. As to the last mechanism, p27 is ubiquitylated and then degradated by SCF^{Skp2}complex.

Skp2 belongs to the F-box proteins, the variable subunits that dictate the specificity of SCF complexes. It has been shown to be required for G1/S transition. Control of cell cycle progression by Skp2 has been linked to its ability to control the levels of the CDK inhibitor p27.

Although there are different reports of overexpression of Skp2 protein in human haematological and solid cancers so far there are no studies regarding the clinical significance or the biological behaviour of Skp2 expression in human thyroid carcinomas.

The aim of the present study was to determine the role of Skp2 in thyroid cancer development and in the regulation of the p27 expression in thyroid cancer cells, addressing the following objectives:

- to analyse the expression level of Skp2 in thyroid cancer cell lines and primary tumors;

- to determine the effect exerted by Skp2 on p27 expression and cell proliferation;

- to identify the signalling pathway which affect the Skp2/p27 axis in cancer cells.

MATERIALS AND METHODS

Clinical Samples.
Primary thyroid carcinomas and their paired adjacent normal glandular tissues were obtained from patients who underwent surgery from 1993 to 2000, at the Chirurgia B, INT Fondazione G Pascale, Napoli, Italy.

Cell Culture.
Cell lines have been established from PTC: [NPA (Ohta et al. 1997), BHP2.7, BHP10.3, BHP17.10 (Pang et al. 1989), TPC-1 (Tanaka et al. 1987), BC-PAP (Fabien et al. 1994)]; FTC [WRO (Estour et al. 1989)]; ATC [FB-1 (Fiore et al. 1997), FRO (Fagin et al. 1993), ARO (Ohta et al. 1997)]. Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc., Grand Land, NY) supplemented with 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin-streptomycin (Life Technologies, Inc.), in a CO_2 incubator (5% CO2 in air) at 37°C. Control thyroid cells were provided from Dr. F. Curcio (University of Udine, Italy) and grown as described (Curcio et al. 1994).

RNA and DNA Isolation and RT-PCR.
Total RNA and high molecular weight genomic DNA were prepared as described (Chomczynski & Sacchi, 1987; Sambrook et al. 1992). Semiquantitative RT-PCR amplification was performed by 24 cycles, using the following primers: Skp2 sense primer, GCTGCTAAAGGTCTCTGGTGT; Skp2 antisense primer AGGCTTAGATTCTGCAACTTG; actin sense primer GTCAACGGATTTGGTCTGTATT; actin antisense primer AGTCTTCTGGGTGGCAGTGAT.

Quantitative reverse transcription real-time PCR (Q-RT-PCR) and quantitative real-time PCR (Q-PCR).
Q-RT-PCR and Q-PCR were performed using the Power SYBR Green PCR Master Mix in an ABI Prism 7300 thermocycler (Applied Biosystems, Foster City, CA, USA), in presence of 0.4 μM primers, in a total volume of 25μl. cDNAs were synthesized from 5 mg of total RNA by Superscript In all real-time PCR experiments we run the final dissociation stage to generate a melting curve for verification of amplification product specificity. All primers were designed and tested for their specificity using the Primer Expressv1.5 (Applied Biosystems). For RNA analysis, primers were designed to span two adjacent exons in the Skp2 gene (exons 4 and 5, respectively) to avoid amplification of contaminating genomic DNA sequences. The target amplicon was 120 bp.
Oligonucleotides sequences used as primers in the Q-RT-PCR experiments were the following: Skp2 forward (on exon 4): 5'-CGCTGCCCACGATCATTTAT-3', Skp2 reverse (on exon 5): 5'-

TGCAACTTGGAACACTGAGACA-3', β-actin forward: 5'-TGCGTGACATTAAGGAGAAG-3', β-actin reverse: 5'-GCTCGTAGCTCTTCTCC-3'.
The expression levels of Skp2 mRNA in each sample was normalised on the basis of the respective actin content and recorded as a relative expression level.
For Skp2 gene dosage analysis the target amplicon was 98 bp spanning exon 5 and intron 5 of Skp2 gene. Oligonucleotides sequences used as primers in the Q-PCR genomic DNA quantification experiments were the following:
Skp2 forward (exon 5): 5'-CTCCACGGCATACTGTCTCA-3', Skp2 reverse (intron 5): 5'-ACTCTGCCTGCCACTCACTT-3', β-actin forward: 5'-TGCGTGACATTAAGGAGAAG-3', β-actin reverse: 5'-GCTCGTAGCTCTTCTCC-3'.
Each sample was run three times and each PCR experiment included two non-template control wells. The relative amounts of mRNA or DNA were calculated by the comparative cycle threshold (CT) method by Livak and Schmittgen (Livak and Schmittgen 2001) and subsequently normalized by β-actin expression.

Fluorescence In Situ Hybridisation (FISH)
FISH analysis was performed on 10 Formalin-Fixed Paraffin-Embedded samples (5 PTC, 2 FTC, 3 ATC). The copy number status of the SKP2 locus was determined by using two different probes: (1) three BAC clones covering the gene (RP11-36A10, RP11-262E5 and RP11-624K2) that were labelled with dUTP-SprectrumOrange (Vysis Inc., DownersGrove, IL; USA) and (2) three BAC clones as control probe (RP11-215J12, RP11-397H14 and RP11-620I22) covering the 5q31 region that were labelled with dUTP-SpectrumGreen (Vysis Inc., DownersGrove, IL; USA). The BAC clones used were designed according to the Ensembl database (www.ensembl.org). The slides were deparaffinized, boiled in a pressure cooker with 1mM EDTA, pH 8.0 for 10 min, and incubated with pepsin at 37ºC for 30 min. The slides were then dehydrated. The probes were denatured at 75ºC for 2 min after overnight hybridization at 37ºC in a humid chamber. Slides were washed with 0.4 X SSC and 0.3% NP40. FISH evaluation was performed by two investigators with no previous knowledge of other genetic, clinical or IHC results. Scoring of fluorescence signals was carried out in each sample by counting the number of single copy gene and control probe signals in an average of 130 (60-210) well-defined nuclei. Gain or Amplification status was counted as the ratio (in > 5% of tumour cells) of red/green signals. The cut-off values for the copy number changes were obtained from the analysis of normal adjacent cells in each experiment.

Immunohistochemistry.
Immunohistochemical studies of Skp2 and p27 in thyroid carcinoma were

performed using the avidin-biotin-peroxidase method (LSAB kit; DAKO, Glostrup, Denmark) on formalin-fixed, paraffin-embedded tissues as described previously (Signoretti et al. 2002; Li et al. 2004). All sections were counterstained with hematoxylin. p27 was scored by observing 500 cancer cells in at least ten high-power fields and were classified as positive (staining in >40% of cells) or negative (staining in <40% of cells) as described previously. The expression of Skp2 was graded as high (>20% of tumour cells showed strong or diffuse immunopositivity), or low (<20% of the tumour cells showed weak, focal immunopositivity or absence of staining).

Western Blot and Antibodies.
Whole cell extracts were prepared from cultured cells by homogenizing cells in NP-40 lysis buffer (10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40) containing protease inhibitors. Western blot analysis was carried out by standard methods using whole cell extracts. The antibodies used in this study were: anti-p27 (Transduction Laboratories, Lexington, KY, USA); anti- ERK and anti-phospho-ERK (anti-T202/Y204) (New England Biolabs, Lake Placid, NY). The anti-SKP2 and phospho-T187 p27 antibodies were from Zymed Laboratories Inc. (San Francisco, CA, USA). Anti-HA and anti-βtubulin were from Santa Cruz Biotechnology, Inc. (California, USA).

Transfection Assays
Human Skp2 cDNA was a gift of Dr. Giulio Draetta (Milan, Italy). One x 10^5 cells were plated onto a 6-well multiwell plate and allowed to attach for 24 h. Transfection was done with Fugene 6 reagent (Roche Applied Biosciences, Basel, Switzerland). Forty-eight hours after transfection, cells were treated with 800 μg/mL G418 (Invitrogen, Carlsbad, CA) for 2-3 weeks as a selective marker. Surviving cells were dispersed in a 24-well multiwell plate and several stably transfected cell clones were obtained after 2–3 more weeks. Each clone was screened for Skp2 expression by Western blot analysis.

Northern blot analysis
Northern blot analysis was performed according to a standard procedure.50-51 In brief, equal amounts of total RNA (20 μg/lane) were denatured and resolved electrophoretically through formaldehyde-agarose gels. The RNA was transferred onto a nylon membrane and cross-linked by UV irradiation. Human p27 cDNA was labeled with ^{32}P-dCTP using a random primer labeling kit (GE Healthcare, Orsay, France), and hybridization was performed at 42°C in the presence of 50% formammide.

RNA interference
Anti-sense experiments targeting Skp2 were performed as described previously (Yokoi et al. 2004). Oligonucleotides containing phosphorothioate backbones

were synthesized (Invitrogen, Rockville, MD, USA): AS, 5'-CCTGGGGGATGTTCTCA-3' (the anti-sense direction of human Skp2 cDNA nucleotides 180 to 196) and SC, 5'-GGCTTCCGGGCATTTAG-3' (a scrambled control for AS). Oligonucleotides (AS or SC), were delivered into ATC cells at the final concentration of 200 nmol/L each, using oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested by trypsinization 24 or 48 hours after transfection for growth and viability assays.

Virus generation and infection

The Human p27 (NM_004064) MISSION shRNA set (5 individual hairpins individually cloned into pLKO.1-puro) (Sigma, St. Louis, MO, USA) was used to generate lentiviral particles in HEK 293T packaging cells. Subconfluent HEK293T cells were cotransfected with 13 µg of p27 MISSION shRNA set, 18 µg of pCMV-deltaR8.91, and 12 µg of pCMV-VSVG per 100mm tissue culture plate by calcium phosphate precipitation (Zufferey et al. 1997). Starting 48 hours after transfection, supernatants were collected at 8-hour intervals, filtered and used for three rounds of transduction of WRO cells in the presence of 8µg/ml of polybrene (Sigma). Transduced cells were lysed after 72 hours from infection, and were analyzed by immunoblot with anti-p27 antibodies. p27 knockdown in transduced WRO cells was determined by immunoblot and compared to untransduced WRO cells, to Mission non-target control transduction virus (Sigma SHC002V) and to Mission TurboGFP™ Control Vector encoding virus (Sigma SHC003V).

***In Vitro* Proliferation Assay and Flow Cytometry Assay.**

Mock-transfected and Skp2-transfected cells were plated at a density of 1.0 x 10^5 cells/well in triplicate and were harvested and counted every day for 6 days. The medium was changed every 48 h. This experiment was repeated three times.

Indirect Immunofluorescence

Cells were grown to subconfluence on coverslips, fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. 5'-bromo-3'-deoxyuridine (BrdU) was added to the culture medium to a final concentration of 10 µM for 2 hours. S-phase cells were identified by use of a 5'-bromo-3'-deoxyuridine Labeling and Detection Kit (Roche Applied Science). Cell nuclei were identified by Hoechst staining. Fluorescence was viewed with a Zeiss 140 epifluorescent microscope equipped with filters that allowed discrimination between Texas red and FITC.

***In Vitro* p27 Protein Degradation Assay.**

Human His-tagged p27 was generated by RT-PCR and cloned into pET21a

plasmid as described (Viglietto et al. 2002). The 6-Histidine tag was at the C-terminus. The p27 degradation assay was performed as described by Pagano and coworker (Pagano et al. 1995). Frozen tissue samples and cell pellets were resuspended in lysis buffer containing 20 mM Tris-HCl (pH 8.5) and 1 mM DTT and subsequently frozen and thawed several times. Lysates were then centrifuged at 13,000 rpm and the supernatant was retrieved and stored at -80°C for the degradation assay. Purified histidine-tagged p27 was incubated at 37°C in a degradation mixture containing 100 µg of cell extract, 50 mM Tris-HCl (pH 8.5), 5 mM MgCl2, 1 mM DTT, 2 mM ATP, 10 mM creatine phosphokinase (Sigma), 10 mM creatine phosphate (Sigma), and 5 µM ubiquitin (Sigma). p27 degradation rate was analyzed by immunoblotting with an anti-p27 antibody.

RESULTS AND DISCUSSION

1. p27 regulation in thyroid cancer

The cyclin-dependent kinase inhibitor, p27, is inactivated in thyroid cancer either through loss of expression or cytoplasmic mislocalization (Baldassarre et al. 1999, Vasko et al. 2004; Motti et al. 2005). Although cytoplasmic delocalization in thyroid cancer cells has been shown to involve the activity of the PI3K/AKT pathway, it is as yet unclear what pathway promotes p27 loss in thyroid cancer.

Therefore, my Doctorate project focused on molecular mechanisms implicated in p27 down-regulation observed in thyroid cancer cells.

In order to further shed light on p27 regulation in thyroid cancer, we analysed specimens derived from thyroid carcinomas by western blot for expression of p27 protein, and by northern blot for p27 mRNA levels. The tumours analysed were PTC (62), FTC (29) and ATC (8). Ten different non-neoplastic thyroid glands and 10 FA served as controls of normal and benign tumour tissue. In agreement with previous studies (Resnick et al. 1998, Baldassarre et al. 1999, Kondo et al. 2006), we found that p27 was expressed at high level in the follicular cells from normal glands and remained sufficiently elevated in most FA (9/10). However p27 expression was reduced in ~60% of carcinomas. The bar graph in figure 8A shows the levels of p27 protein (upper panel) and mRNA (lower panel), observed in normal thyroid gland (bars 1-10) and FA (bars 11-20) and in 99 thyroid carcinomas of different histotypes (bars 21-82, PTC; bars 83-111, FTC; bars 112-119, ATC), quantified through Phosphorimager scanning and expressed as arbitrary units. As shown in the figure, p27 expression is severely reduced in 36/62 PTC (58%), in 23/29 FTC (79%) and in 5/8 ATC (63%). Conversely, a significant loss of p27 mRNA occurred only in 18/99 specimens (18%) of thyroid carcinomas analysed (10 PTC, 7 FTC, 1 ATC). As expected, in most cases in which p27 mRNA expression was severely reduced the levels of p27 protein were also consistently reduced. Figure 8B shows representative western (upper panel) or northern (middle panel) blot analysis of p27 expression in normal thyroid tissue (lane 1) or thyroid carcinomas (lanes 2-10). In conclusion, the comparison between the data obtained from northern and western blots, reveal that in a limited number of neoplasms, down-regulation of p27 expression occurs at the mRNA level. On the other hand, for most thyroid carcinomas (~65%), we found no consistent correlation between the levels of p27 mRNA and protein.

The failure to find a consistent correlation between the levels of p27 mRNA and protein in the majority of thyroid carcinomas suggest that mechanisms acting at the protein level are likely responsible for the downregulation of p27 expression observed in thyroid cancer.

This is in agreement with the observation that MG132 regulates p27 levels in low expressing thyroid cancer cells. We used six cell lines established from human thyroid carcinomas (PTC: TPC1, NPA; FTC: WRO; ATC: FRO, ARO

and FB1). Treatment of these cells with the proteasome inhibitor clearly induced upregulation of p27 protein levels, while Skp2 levels were reduced. Figure 8C shows an experiment with three representative cell lines.

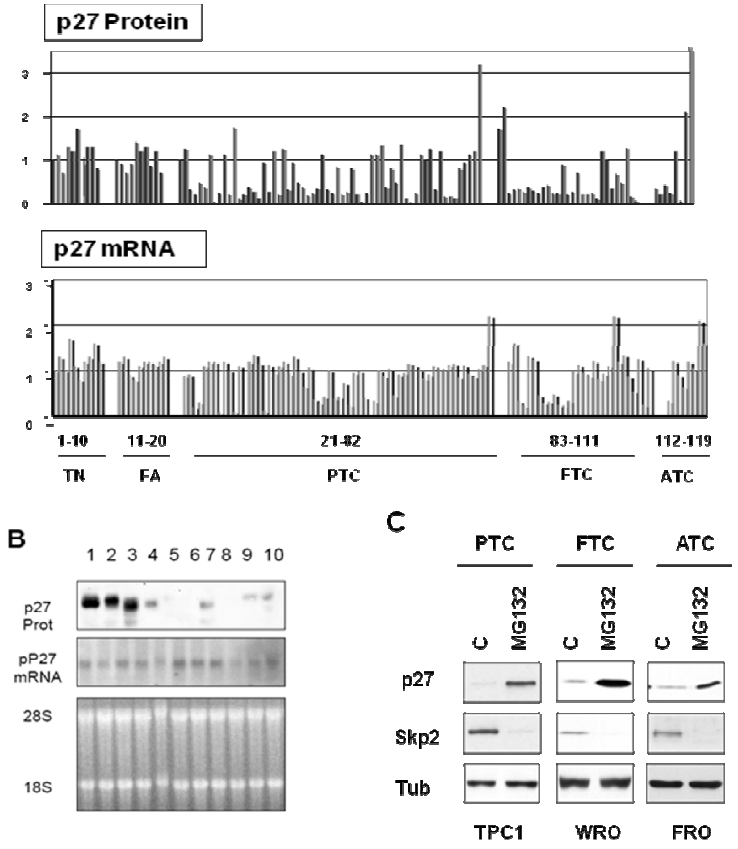


Figure 8. Loss of p27expression in thyroid cancer occurs by increased protein turnover. A. Expression of p27 protein and mRNA in thyroid tumours. Each bar represents the relative protein or mRNA expression measured in a single specimen. Expression of p27 was determined by western blot (for protein levels) and by northern blot (for RNA levels), quantified by densitometric analysis with the ImageQuant software and expressed as arbitrary units. **B.** Representative western blot (upper panel) and northern blot (middle panel) analysis of p27 expression in normal tissue (lane 1) or thyroid carcinomas (lanes 2-10); lower panel, ethidium bromide staining of 28S/18SZrRNAs were used to ensure equal loading of total mRNA. **C.** Western blot analysis of p27 and Skp2 expression in cells treated with DMSO (C) and proteasome inhibitor MG132 (50 μM for 6 hours).

Altogether, these data suggest that p27 down-regulation in thyroid carcinomas is prevalently obtained through alteration of p27 protein turnover. Therefore, we investigated whether Skp2 was involved in p27 degradation in thyroid cancer cells.

2. Expression of Skp2 in thyroid cancer cell lines.

On the basis of data described above, we decided to determine the role of Skp2 in thyroid carcinogenesis. We analyzed expression of Skp2 protein in 10 cell

lines derived from human thyroid tumours: 6 cell lines were derived from PTC (NPA, BHP2.7, BHP10.3, TPC-1, BHP17.10, B-CPAP), one from FTC (WRO) and three from ATC (FB-1, ARO, FRO) (fig. 9A). The primary P5 cells were used as control of normal human thyroid cells (TN).
Western blot analysis demonstrated that Skp2 expression was moderately increased in 4/9 PTC cell lines (NPA, BHP10.3, BHP17.10 and B-CPAP), and markedly overexpressed in 2 ATC cell lines (FRO, ARO). Subsequently, we examined Skp2 mRNA expression by Northerm blot (not shown) and quantitative RT-PCR in a selected panel of thyroid cancer cell lines (NPA, B-CPAP, TPC-1, ARO, FRO and FB-1) (fig. 9B).
We found a good correlation between Skp2 mRNA and protein expression in most cell lines analysed. In fact, the lines that showed low levels of Skp2 protein (FB-1, TPC-1) also showed low amount of mRNA whereas the cell lines with increased Skp2 protein levels showed enhanced amount of Skp2 mRNA (B-CPAP, FRO and ARO).

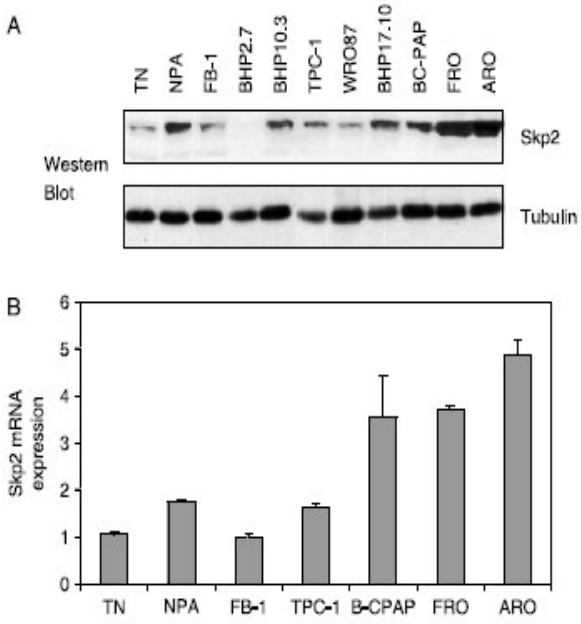


Figure 9. Skp2 expression in human thyroid cancer cell lines. A. Immunoblot analysis of Skp2 in human thyroid cancer cell lines. TN, normal thyroid cell line (P5); NPA, BHP2.7, BHP10.3, TPC-1, BHP17.10 and B-CPAP cell lines were derived from papillary carcinomas (PTCs), WRO from follicular carcinoma (FTC), FB-1, ARO and FRO cells were derived from ATC. Antibodies to β-tubulin were used for normalization. **B.** Skp2 relative mRNA expression by Q-RT-PCR. Control, RNA from P5 cells (TN).

3. Expression analysis of Skp2 in thyroid tumours.

Although the results described above suggest that Skp2 may play a key role in the process of thyroid carcinogesesis, it is necessary to note that the various thyroid cancer cell lines used in this study could not be the most relevant models of primary thyroid tumours. Therefore, we extended our study to the expression of Skp2 in primary carcinomas. To this aim, we performed immunostaining for Skp2 protein in 12 cases of TG, 10 FA and 51 cases of thyroid carcinomas (19 PTC, 20 FTC and 12 ATC). The incidence of Skp2

expression in thyroid disease is summarized in fig. 10A. We found that Skp2 is not detectable in thyrocytes from normal glands (4/4) and in hyperplastic disease such as goiters (12/12), and is expressed only in 1/10 FA with few (~10%) focally distributed nuclear-positive tumour cells (fig. 10B). In contrast, Skp2 is overtly overexpressed in 26/51 (~51%) of malignat thyroid carcinomas. Thyroid carcinomas were classified as positive for Skp2 expression when showing strong diffuse nuclear staining in several cells/high power fields (cut-off set at >20% of tumour cells). Positive Skp2 expression was significantly higher in the carcinoma samples than either goiters or adenomas (carcinoma *versus* goiter, P<0.001; carcinoma *versus* adenoma, P<0.05; Fisher's exact test). Among the different types of thyroid carcinomas, Skp2 expression was observed more frequently in ATC (83%, 10/12) than PTC (42%, 8 of 19) or FTC (40%, 8/20) (P<0.05; Fisher's exact test).

These results are in agreement with previous studies that have shown Skp2 overexpression in different cancerous tissues, with a striking correlation between Skp2 overexpression and grade of malignancy, lymph node metastasis and poor prognosis (Gstaiger et al. 2001, Hershko et al. 2001, Kudo et al. 2001, Latres et al. 2001, Chiarle et al. 2002, Masuda et al. 2002, Signoretti et al. 2002, Li et al. 2004), and shed light on the molecular alterations that occur during thyroid carcinogenesis.

A

Tumor types	Low Skp2 expression[a]	High Skp2 expression[a]	Total (%)
Goiter[b]	12	0	0/12 (0)
Follicular[c] Adenoma	9	1	1/10 (20)
Papillary[d] Carcinoma	11	8	8/19 (42)
Follicular[e] Carcinoma	12	8	8/20 (40)
Anaplastic Carcinoma	2	10	10/12 (83)

B

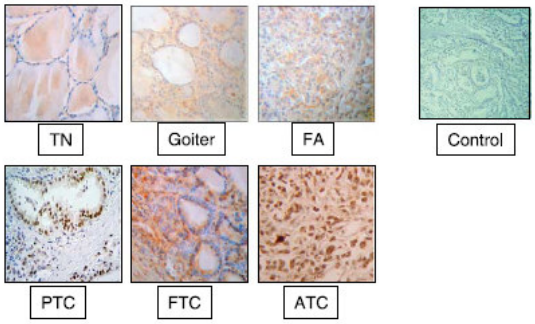


Figure 10. Skp2 expression in human primary thyroid cancer. **A.** Expression of Skp2 in thyroid tumours. [a] The expression of Skp2 was graded as high (>20% of tumour cells with strong or diffuse immunopositivity), or low (<20% of the tumour cells with weak, focal immunopositivity or absence of staining). [b] Significant; goiter *versus* carcinomas: P=0.0008 (Fisher's exact test). [c] Significant; adenoma *versus* carcinomas: P=0.032 (Fisher's exact test). [d] Significant; papillary *versus* anaplastic carcinomas: P=0.031 (Fisher's exact test).[e] Significant; follicular *versus* anaplastic carcinomas: P<0.027 (Fisher's exact test). **B**. Representative immunostaining of Skp2 in normal and pathological thyroid tissue. Serial formaline-fixed paraffine-embedded sections from normal thyroid tissue (TN), goiter, FA, PTC, FTC, ATC were analyzed for Skp2 expression. Most follicular epithelial cells from normal glandular tissue, goiter and FA are negative for Skp2 expression; several cancer cells show intense nuclear Skp2 expression in PTC, FTC, ATC. Magnification, 100X. C. Semiquantitative RT-PCR analysis of Skp2 mRNA expression in thyroid tumours. Twenty-four cycles RT-PCR products were separated by agarose gel, transferred to nylon membrane and hybridized. Actin expression was used as a loading control. TN, normal thyroid; FTA, follicular adenoma; FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; ATC, anaplastic thyroid carcinoma; C, blank control without DNA for PCR reaction.

4. p27 versus Skp2

The relationship between Skp2 and p27 protein expression was examined in 68 cases of thyroid tumours. Expression of p27 in thyroid tumour tissues was evaluated by immunohistochemistry with anti-p27 monoclonal antibody (Fig. 11A and B). Thyroid cancer cases showed three major patterns of Skp2/p27 expression. Eighteen tumours (26%) were characterized by low p27 and high Skp2 levels. Twenty-four tumours (35%) expressed high p27 and low Skp2 levels. In 20 tumours (29%) both low Skp2 and p27 levels were observed. Only 6 tumours expressed both high levels of Skp2 and p27. Fisher's exact test indicated that the percentage of high Skp2 expressors was significantly greater in the low p27 group than in the high p27 expression group ($P<0.05$). Benign tumours that presented low expression of Skp2 (12 TG and 6/8 FA) showed high nuclear expression of p27 wheras 18/24 carcinomas with high Skp2 expression showed reduced levels of p27 protein. Figure 11B shows a representative immunostaining. Results were also confirmed by immunoblot (fig. 11C). To determine the contributon of T187 phosphorylation to p27 degradation, we also determined p27 phosphorylation at T187 in the same samples of primary thyroid carcinomas (Fig. 11C). We found that most samples expressed high levels of Skp2 protein and thus lacked p27 and T187 phosphorylation, whereas a few samples (#10 and 13) showed low levels of Skp2 and high levels of p27. In these samples the relative amount of T187-phosphorylated p27 was rather low.

Conversely, in samples #11 and 12, that presented low levels of p27 protein, the signal detected by the phospho-T187 antibody indicated that a great amount of p27 protein in those samples was phosphorylated. These studies indicated that Skp2 expression was inversely correlated with p27 levels and that both Skp2 levels and the different extent of T187 phosphorylation of p27 may contribute to determine p27 expression in thyroid cancer cells.

Because the reduction in p27 protein is brought about by ubiquitin-proteasome-mediated degradation, we also examined p27 degradation activity in these tissues. We compared the ability of proteasome extracts deriving from 3 different ATC (that showed high levels of Skp2 protein) to degrade recombinant p27 in an *in vitro* degradation assay with that of 2 different PTC with low Skp2 expression. Extracts from ATC showed higher p27 degradation activity in agreement with the very low level of p27 protein expression; conversely, extracts from PTC showed much lower p27 degradating activity (compare kinetics of p27 degradation rate in # 8 and #14 in Fig. 11D).

The results here reported indicate that enhanced p27 degradation observed in thyroid cancer can be attributed to Skp2 overexpression. Moreover, the existence of some thyroid cancer cases that showed low expression of Skp2 and p27 implied that other systems might also regulate p27 protein expression. Recent studies showed that cyclin-dependent kinase subunit 1 (Cks1) was required for the ubiquitination of p27 by bridging Skp2 and its substrate, p27, *in vivo* and *in vitro* (Ganoth et al. 2001; Spruck et al. 2001).

On the other hand, p27 degradation may be accomplished through Skp2-independent mechanisms (Hara et al. 2001; Kamura et al. 2004).

A

	Low p27 expression[b]	High p27 expression[b]	Total
Low Skp2 expression[c]	20	24	44
High Skp2 Expression[c]	18	6	24
Total	38	30	

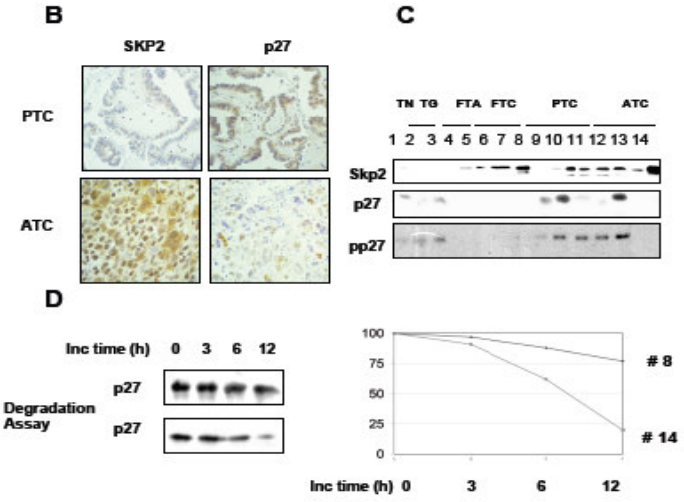


Figure 11. Correlation between Skp2 and p27 expression in thyroid primary tumours. A. Correlation between the expression of Skp2 and p27 in thyroid tumours (P=0.023) with two-tailed Fisher exact test. [b] Cells were classified as p27-positive if staining was observed in >40% of observed cells, or p27-negative if staining was observed in <40% of observed cells. [c] Cells were classified as as Skp2-positive if staining was observed in >20% of observed cells. **B.** Immunostaining of Skp2 and p27 in a papillary tumour (PTC) that show low Skp2 and high p27 expression; immunostaining of Skp2 and p27 in an anaplastic tumour (ATC) with high Skp2 and low p27 expression. **C.** Reciprocal expression of Skp2 and p27 in human primary thyroid carcinoma by western blot. TN, normal thyroid tissue; TG, goiter; FTA, follicular adenoma; FTC, follicular carcinoma; PTC, papillary carcinoma; TC, anaplastic carcinoma. **D.** Rate of p27 degradation in extracts from sample 8 or 14. One µg of recombinant p27 was incubated at 37°C with 100 µg of proteasome extracts from tumours 8 and 14 for 0, 3, 6 or 12 hours, respectively, and the subsequent immunoblot analysis revealed the amount of intact p27 protein in different conditions. Half-life of p27 protein was of approximatively 7 hours in sample 14 and > 12 hours in sample 8.

5. Skp2 is necessary for p27 degradation and cell proliferation in ATC cells

In order to determine the effect of Skp2-dependent degradation of p27 in thyroid cancer cells, we made use of antisense technology. ATC cells, FRO and ARO, were transfected with Skp2 antisense or control oligonucleotides (200 nM). After 48 hours, cells were incubated with 10 μM BrdU for 2 hours and processed for western blot and indirect immunofluorescence. As shown in Figure 12A, we were able to reduce Skp2 expression by >70% in ARO cells and almost 90% in FRO cells, whereas the same dose of control sequence-scrambled oligonucleotides (Fig. 12A, C) had no effect on the cellular levels of Skp2. In parallel with the decrease in Skp2 expression, the treatment of ATC cells with anti-Skp2 antisense oligonucleotides induced a marked induction of p27 protein levels. Such increase in p27 elevels in AS-treated WRO cells results in increased binding of p27 to Cdk2 and decreased activity of the kinase using the histone H1 as substrate (not shown).

Finally, we found that the capacity to incorporate BrdU of ARO and FRO cells was markedly reduced in cells that were administered antisense oligonucleotides (Fig. 12B, AS) compared to untransfected (Fig. 12B, C1), mock-transfected ATC cells (Fig. 12B, C2) or cells transfected with control oligonucleotides (Fig. 12B, C3).

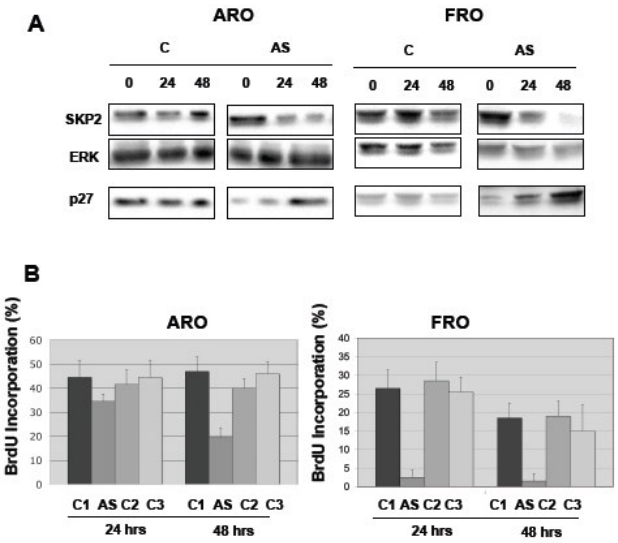


Figure 12. Suppression of Skp2 protein expression inhibits proliferation of human ATC cells. To suppress the expression of SKP2 in ATC cells, ARO and FRO cells were plated onto glass coverslips in DMEM containing 10% FBS and then transfected with SKP2 antisense or scrambled control oligonucleotides with oligofectamine. After 24 and 48 h, cells were incubated with 10 μM BrdU and processed for western blot (**A**) or indirect immunofluorescence (**B**). **A.** Western blot analysis of cyclin SKP2 expression in the presence of control (C) or anti-SKP2 antisense oligonucleotides (AS). **B.** Graphs indicate the percentage of BrdU-positive cells. BrdU positive cells were evidentiated using FITC-conjugated secondary antibodies. C1, untransfected cells; AS, anti-Skp2 oligonucleotide-transfected cells; C2, mock-transfected cells; C3, cells transfected with an oligonucleotide of scrambled sequence.

6. Skp2 promotes cell proliferation in thyroid carcinoma cells

To further investigate the role played by Skp2 in the control of thyroid cell cycle progression, we transfected WRO cells with a plasmid that carry the cDNA encoding human Skp2 cDNA. Transfected cells were selected in G418-containing medium and several WRO-neo and WRO-Skp2 clones were isolated and expanded. The presence of the exogenous Skp2 protein in transfected cells was detected by western blot analysis (Fig. 13A). Two different WRO-neo (cl.2 and cl.9) and three different WRO-Skp2 clones (cl.3, cl.5 and cl.17) were selected for biological studies. Since the same data were obtained with the three clones in all our experiments, we will refer to them as "WRO or WRO-Skp2 cells". As expected, forced expression of Skp2 protein in WRO cells promoted increased binding to T187-phosphorylated p27 (not shown) and reduced p27 levels (Fig. 13A).

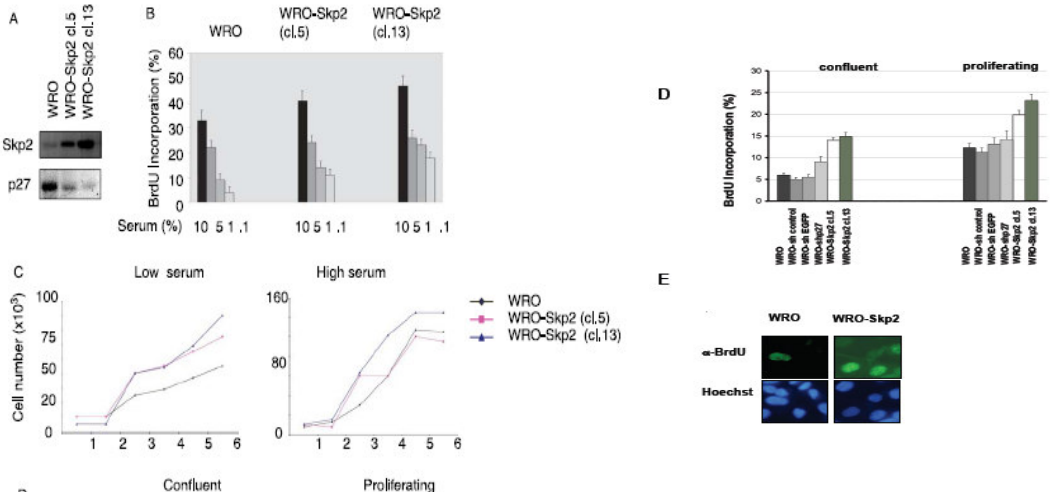


Figure 13. Skp2 promotes proliferation of thyroid cancer cells. A. Effects of adoptive Skp2 expression on the growth rate of thyroid cancer cells. A. Skp2 and p27 expression in WRO and WRO Skp2 (Cl.5 and Cl.13) cells. **B.** WRO and WRO-Skp2 (Cl.5 and Cl.13) cells were plated onto glass coverslips in complete DMEM and, after 24 h, the medium was replaced with DMEM containing 10, 5, 1 and 0.1% FBS for further 24 h. Subsequently, cells were incubated with 10 μM BrdU and processed for indirect immunofluorescence. **C.** Growth rates of parental and Skp2-transfected WRO cells in low or high serum content. WRO and WRO-Skp2 (Cl.5 and Cl.13) cells were plated in DMEM supplemented with 1% (low serum) or 10% (high serum) FBS and counted every day for 6 days. A representative experiment of three is reported. **D.** WRO and WRO-Skp2 (Cl.5 and Cl.13) cells were plated onto glass coverslips in complete DMEM medium, at <25% (low density) or >80% (high density) and allowed to grow as described in the text.. BrdU-positive cells were evidentiated using FITC-conjugated secondary antibodies. Cell nuclei were identified by Hoechst staining. Fluorescence was visualized with a Zeiss 140 epifluorescent microscope. **E.** A representative BrdU incorporation experiment. Magnification, 100X.

We investigated whether the constitutive expression of Skp2 modified specific proliferative parameters of thyroid cells including proliferation rate, serum requirement and contact inhibition of WRO cells. To this aim, WRO or WRO-Skp2 cells were plated onto glass coverlips and, after allowing cells to attach for 24 hours in complete medium, the medium was changed and the cells were grown in DMEM containing 10%, 5%, 1%, and 0.1% FBS for further 24 hours, incubated with 10 μM BrdU and processed for indirect immunofluorescence. As shown in the graphs of Figure 13B, that indicate the percentage of BrdU positive cells, BrdU incorporation of WRO-Skp2 cells was higher than that of parental WRO and WRO-neo cells. This effect was more evident for cells grown in serum as low as 1% and 0.1%. Figure 13B reports a representative experiment.

Although BrdU incorporation is a good measure of the rate of DNA synthesis, it does not measure cell division. To further characterize the effect of Skp2 overexpression on the proliferative potential of thyroid cancer cells, we measured the growth rates of parental and Skp2-transfected WRO cells in low or high serum content.

WRO and WRO-Skp2 cells were plated in DMEM supplemented with 1% (low serum) or 10% (high serum) serum and counted every day for 6 days. A representative experiment of three is reported in Figure 13C. As shown in the Figure, the growth rate of WRO-Skp2 cells was higher compared with parental WRO cells, especially when grown in low serum-containing medium. Moreover, when grown at confluence, WRO-Skp2 cells incorporated more BrdU compared to WRO cells, which suggested that Skp2 relieved the growth arrest induced by high density culturing in WRO cells (Figure 13D).

Skp2 interacts with several cell cycle regulatory proteins (Patton et al. 1998; Zhang et al. 1995; Sutterluty et al. 1999; Philipp-Staeli et al. 2001). To determine whether the stimulation of proliferation induced by overexpression of Skp2 in WRO cells was entirely mediated by the reduction of p27 levels, we silenced p27 expression by use of lentiviral transduced shRNA to p27 both in confluent and proliferating WRO cells. Although interference with p27 expression was complete in both confluent and proliferating WRO cells (not shown), we observed a partial but reproducible increase in the BrdU uptake of transduced confluent WRO cells compared with untransduced WRO cells or transduced with Mission non-target control transduction virus (Sigma SHC002V) (Figure 13D). This proliferative effects induced by interferring with p27 expression was observed only in confluent but not in proliferating WRO cells (Figure 13D). These findings suggest that the effect on the proliferation of WRO cells exerted by Skp2 was mediated only in part by the control of p27 expression and that the regulation of other cell cycle regulatory proteins by Skp2 must contribute to thyroid cancer cell proliferation, especially in proliferating cells.

In summary these data indicates that Skp2 can act as a rate-limiting factor for G1/S transition in cultured thyroid cancer cells and its unrestrained expression removes the serum-dependency constraint and impairs contact-inhibition-induced G1 arrest.

7. Mechanism of Skp2 overexpression in thyroid cancer

7.1 Gene amplification

Previous work has shown that the Skp2 gene mapping at 5p13 is frequently target of amplification in lung cancer (Yokoi et al. 2002; Yokoi et al. 2004; Coe et al. 2005), in glioblastoma (Saigusa et al. 2004) and in carcinoma of the biliary tract (Sanada et al. 2004). Interestingly, an increase in the copy number of chromosome 5 involving the locus 5p13, where the Skp2 gene has been assigned, was reported to occur in FTC, in Hurthle carcinomas (Hemmer et al. 1999; Wada et al. 2002) and in ATC cell lines including ARO (Foukakis et al. 2005). Thus, we examined the amplification status of Skp2 gene in thyroid carcinoma cell lines (Fig. 14A). Genomic DNA extracted from 6 thyroid cancer cell lines and from control normal human lymphocytes (Fig. 14A, control) was analysed by Q-PCR as described in Materials and Methods.

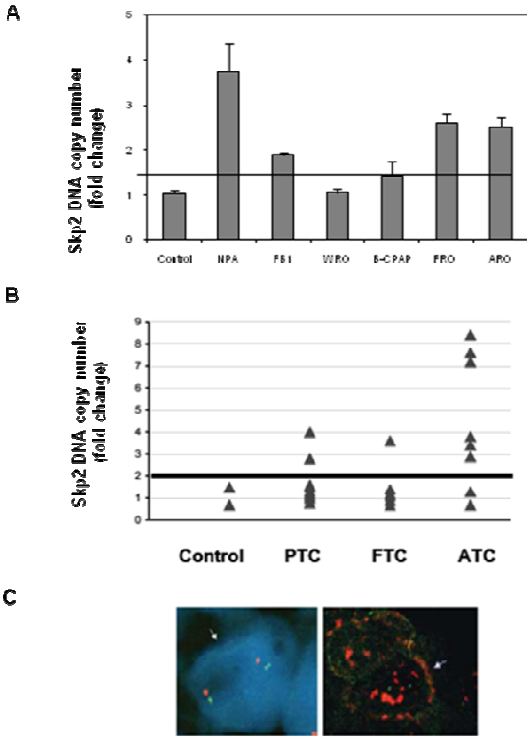


Fig. 14. Skp2 overexpression by gene amplification. Skp2 relative gene number by Q-PCR in thyroid cell lines (**A**) and in primary thyroid cancer (**B**). Control, DNA from peripheral blood lymphocytes. **C.** FISH analysis of primary thyroid carcinomas. Left, the wite arrow points to a cell showing the normal complement of Skp2 gene (red); right, the white arrow points to a cell showing multiple signals of Skp2 (red).

The DNA copy number of the Skp2 gene was considered increased when the arbitrary values obtained by the quantitative PCR analysis (after normalization with actin levels), were greater than two-fold the value obtained normal diploid

human lymphocytes. Using these parameters, our results indicated that at least three cell lines presented increased copy number of the Skp2 gene (NPA, FRO, ARO) in comparison to genomic DNA from normal lymphocytes or from other cell lines examined. At least two out of the 3 cell lines that presented amplification of the Skp2 gene (ARO, FRO) expressed the highest levels of Skp2 mRNA and protein.

Subsequently, we analysed the amplification status of the Skp2 gene in human thyroid tumours. Therefore, we performed Q-PCR on genomic DNA extracted from 23 thyroid tumours (9 PTC, 6 FTC, 8 ATC) using normal human lymphocytes as control. As with cell lines, DNA copy number of the Skp2 gene was considered increased when the values obtained by Q-PCR analysis with the Skp2 primers, were greater than two-fold the value obtained normal diploid human lymphocytes.

We found that, compared to normal lymphocytes, six out of eight ATC, one of nine PTC and one out of six FTC showed Skp2 gene increased copy number (Fig. 14B). DNA amplification at 5p13 was also evaluated by performing FISH analysis on 10 cases as described in Materials and Methods. FISH analysis demonstrated that the copy number of the Skp2 gene was increased in all three ATC analysed. Conversely, gain of chromosome 5p13 was observed only in one out of 5 PTC and in one out of 2 FTC. See Figure 14C for a representative case.

Therefore, data here reported suggest that gene amplification of the Skp2 locus at 5p13 represents a common event in ATC and may account for the observed increased expression of Skp2 mRNA and protein frequently observed in these tumours. These results confirm cytogenetic data indicating that gain of part or of the entire short arm of chromosome 5, where the Skp2 gene maps, frequently occur in PTC, Hurthle carcinomas and ATC (Hemmer et al. 1999, Wada et al. 2002, Foukakis et al. 2005). However, other mechanisms must be involved in the dysregulation of Skp2 expression in thyroid cancer, since our results indicate that a fraction of thyroid carcinomas overexpresses Skp2 mRNA and protein in the absence of gene amplification of the Skp2 locus. Accordingly, it has been suggested that the expression of Skp2 protein may be regulated at post-transcriptional level (Bashir & Pagano 2004, Wei et al. 2004).

7.2 Post-trascriptional regulation

Since several differentiated thyroid tumours showed high levels of Skp2 in absence of gene amplification, we investigated whether the oncogenes that are implicated in thyroid carcinogenesis, in particular in MAP kinase cascade activation, are responsible for Skp2 expression dysregulation.

We studied the effects of activated RET (Myc-RET/PTC1), H-RAS (HA-HRASV12) and BRAF (Myc- BRAF V600E) on the expression of Skp2 and p27 in thyroid carcinoma cells that are known to be devoid of activated version RET/PTC1 and that are wild type for RAS or BRAF gene sequences (WRO cells).

To this aim, WRO cells were transiently transfected with constructs carrying mutant RET/PTC1, HA-HRASV12 and BRAFV600E and, subsequently, the

expression of endogenous p27 and Skp2 proteins was determined by immunoblot and the activity of MAP kinase cascade was determined with anti pERK antibodies. We found that all three activated oncogenes increased phosphorylation of ERK, enhanced Skp2 expression and decreased p27 protein levels (Fig. 15). These findings suggest that the RAS -> BRAF -> MAP kinase pathway may induce p27 down-regulation by increasing the expression of Skp2.

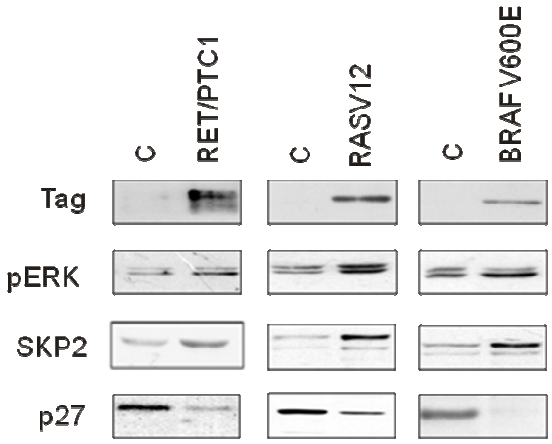


Figure 15. Oncogenic RET/PTC1, H-RASV12 and BRAFV600E regulate p27 and Skp2 expression in thyroid cancer cells. Immunoblot analysis of pMAPK, p27 and Skp2 in WRO cells transfected with activated RET/PTC1, H-RASV12 or BRAFV600E oncogenes. Tranfected proteins were detected with antibodies anti-myc (RET/PTC1, BRAFV600E) or anti-HA (HRASV12).

8. MAP kinase regulates Skp2 and p27 protein stability.

Subsequently, we investigated the mechanisms responsible for MAP kinase dependent regulation of Skp2 and p27 expression in thyroid cancer cells.
The data in the literature concerning the mechanisms involved in MAP kinase-dependent regulation of p27 expression are somewhat controversial. In some studies MAP kinase activation interferes with p27 synthesis whereas in others it interfers with p27 degradation through a RhoA-dependent mechanism (Weber et al. 1997, Hu et al. 1999) or a calpain-dependent pathway (Delmas et al. 2003); in the MCF-7 breast cancer cell line MAP kinase-dependent degradation of p27 apparently requires export from the nucleus through a CRM1-dependent pathway (Foster et al. 2003).
The finding that the block of MAP kinase activity through the MEK inhibitor UO126 (10 μM for 24 hours) reverted p27 down-regulation induced by BRAFV600E (Fig. 16A) and HRASV12 (not shown), indicated that the control of p27 expression exerted by the tyrosine kinase pathway was funnelled mainly through the MAP kinase pathway. Furthermore, treatment of transfected WRO cells with MG132 (50 μM for 6 hours), an inhibitor of the proteasome (Fig. 16B), rescued oncogene-induced p27 down-regulation, indicating that H-RAS- and BRAF-mediated down-regulation of p27 occurs mainly at the posttranscriptional level.

To further investigate the role of MAP kinase pathway in p27 degradation, we used three cell lines (TPC1, WRO, and FRO), which were representative of all histological types of thyroid carcinomas. Results were essentially similar for all cell lines, thus we present the data referring only to one (FRO).

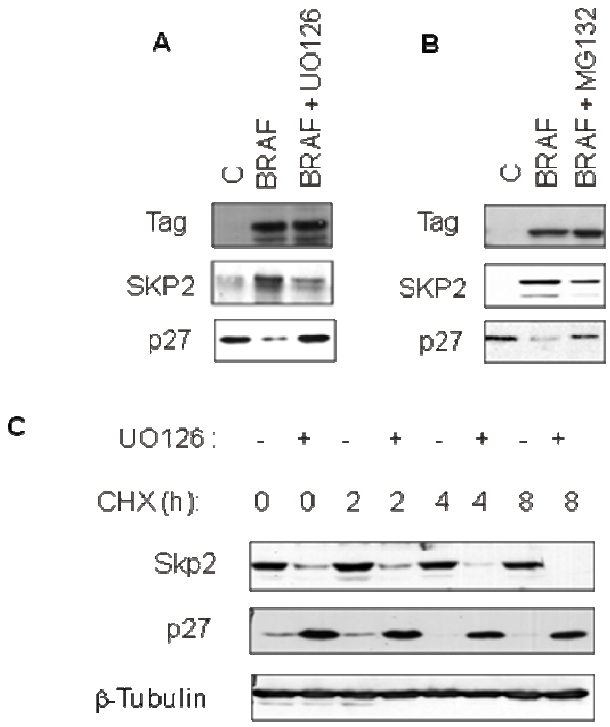


Figure 16. The MAP kinase pathway controls p27 expression by regulating protein stability. A, B. Immunoblot analysis of p27 and Skp2 in WRO cells transfected with BRAFV600E oncogene in the presence of UO126 (A, 10 μM for 24 hours) and MG132 (B, 50 μM for six hours).
C. The half-life of p27 and Skp2 proteins in FRO cells was determined by treating cells with DMSO or U0126 for 24 hours; subsequently the translation of p27 protein was blocked with 10 μM cycloheximide and cell lysates were prepared at different times (2, 4 and 8h), loaded onto SDS PAGE and probed with anti-p27 or anti-β-tubulin antibodies.

To evaluate whether MAP kinase regulated the stability of p27 protein in living thyroid cancer cells, we measured the half-life of p27 protein. To this aim, cells were treated with DMSO or 10 μM U0126 for 24 hours (t=0). At this point, the synthesis of p27 protein was blocked with 10 μM cycloheximide at different times (2, 4 and 8 hours); cell lysates were prepared and equivalent amounts of proteins were loaded on SDS-PAGE and probed with an anti-p27 antibody. As shown in Figure 16C, the disappearance of p27 protein occurred more rapidly in DMSO-treated cells than in U0126-treated cells, indicating a faster degradation of p27 in cells with high MAP kinase activity. After densitometric analysis of the bands, p27 half-life was extimated to be about 2.5- 3 hours in cells with active MAP kinase and of more than 8 hours in cells with low MAP kinase activity. Conversely, the pharmacological inhibition of the MAP kinase

cascade by U0126 drastically reduced the half-life of Skp2 from >8 hours to <4 hours (Fig. 16C).

To further characterize the mechanisms involved in MAP kinase dependent degradation of p27 protein in thyroid cancer cells, we performed the following experiment: cells were treated with U0126 for 24 hours, and then p27 expression was followed by immunoblot on removing the MEK inhibitor from the culture medium for 6, 8 and 24 hours. As observed, MAP kinase inhibition resulted in p27 accumulation, which was reversed, starting after 8 hours and being complete after 24 hours, by removal of UO126 (Figure 17A). Interestingly, p27 expression was mirrored by the expression of Skp2: the expression of Skp2 was drastically reduced by U0126 treatment, which was reversed after 24 hours by removal of UO126 (Figure 17A, SKP2 panel). These results suggest that the MAP kinase cascade regulates p27 through control of Skp2 expression. In a different type of experiment, MAP kinase activity was modulated as in Figure 17A, except that inhibitors of CDK2 (roscovitine), of the CRM1 dependent nuclear export cellular device (Leptomycin B, LMB) or of the ubiquitinproteasome pathway (MG132) were added to the culture medium during the period after removal of the MEK inhibitor.
As shown, p27 down regulation triggered by removal of UO126 was reversed by the addition of the proteasome inhibitor MG132, but not by Roscovitine or LMB (17B). Taken together, these results indicate that in thyroid cancer cells, MAP kinase decreases p27 expression by increasing the p27 degrading activity exerted by the 26S proteasome but this does not require CRM1-mediated nuclear export.

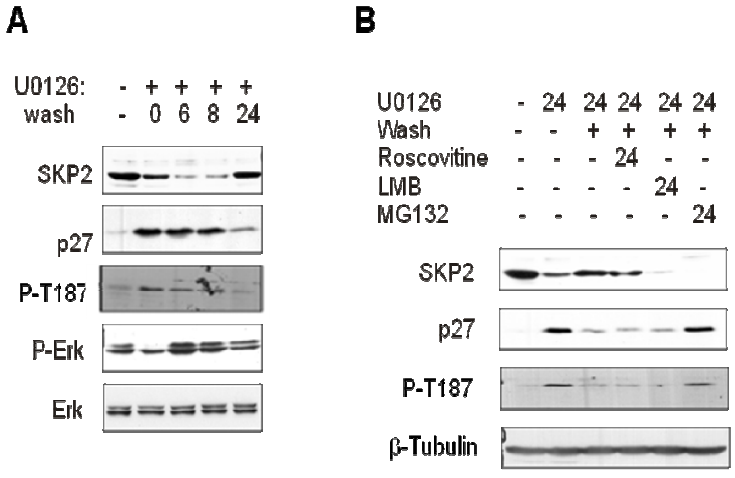


Figure 17. The MAP kinase pathway controls p27 expression by regulating Skp2 protein expression. A. Immunoblot analysis of MAP kinase-dependent p27 expression and (T187) phosphorylation, p45Skp2 expression and ERK1/2 activity in FRO cells. Cells were treated with solvent alone (lane 1) or with 10 μM U0126 for 24 hours (lane 2), washed free of U0126, and cultured for 6, 8, and 24 hours in the absence of U0126. **B.** Cells were treated with solvent alone (lane 1), or with 10 μM U0216 for 24 hours (lane 2), washed from the U0126, and further maintained in culture for 24 hours in the absence or in the presence of Roscovitine, Leptomycin B (LMB) or MG132.

CONCLUSIONS

It is well known that reduced expression of p27 is frequently found in various cancers and correlated with poor survival of cancer patients (Slingerland & Pagano 2000, Philipp-Staheli et al. 2001). Cancer cells express low levels of p27 because of its decreased stability (Slingerland & Pagano 2000, Philipp-Staheli et al. 2001). Although some studies have proposed that the reduced stability of p27 protein observed in human cancer could be due to increased levels of Skp2, the detailed mechanism of abnormal degradation of p27 protein in cancer cells is still unclear. Different groups, including ours, have previously reported that a substantial fraction of thyroid cancer presented reduced p27 expression (Erickson et al. 1998, Baldassarre et al. 1999, Tallini et al. 1999) and, most importantly, this was correlated with lymph node involvement and the presence of distant metastasis (Erickson et al. 1998, Kondo et al. 2006).

We found that in more than two-third of the thyroid cancer samples analysed, the reduction in the levels of p27 protein was not accompanied by a parallel reduction in the levels of the corresponding mRNA; conversely, tumours with low levels of p27 protein presented high rate of p27 degradative activity. We have further extended these data and have reported that the enhanced degradation of p27 protein observed in thyroid cancer can be attributed to Skp2 overexpression. In fact, immunohistochemistry and immunoblot analysis demonstrated an inverse correlation between Skp2 and p27 expression ($n=68$; $P<0.05$). These results suggest that the reduction in p27 protein at least in some thyroid cancer cases may be brought about by high Skp2-mediated degradation.

The mechanism for the overexpression of Skp2 in most cancer is still unknown, though amplification of the Skp2 locus was found in some tumours (lung, bladder and glioblastoma; Yokoi et al. 2002, Sanada et al. 2004, Saigusa et al. 2004, Coe et al. 2005, Ohno et al. 2005). In the work reported here, we showed that Skp2 was amplified in at least three out of six thyroid cancer-derived cell lines and in a significant fraction (75%) of primary thyroid carcinomas analysed, in particular ATC, providing evidence that amplification of the Skp2 locus at 5p13 represents one of the mechanisms capable of enhancing Skp2 expression in thyroid cancer. However, other mechanisms must be involved in the dysregulation of Skp2 expression in thyroid cancer, since our results indicate that a fraction of thyroid carcinomas overexpresses Skp2 mRNA and protein in the absence of gene amplification of the Skp2 locus.

The MAP kinase signalling pathway is known to contribute to the malignant progression of human cancer, though the cause of MAP kinase activation differs among different tumours. In thyroid carcinomas different upstream genetic alterations are implicated in the activation of the MAP kinase signaling cascade. We demonstrate that RET/PTC1-, H-RAS-, or BRAF-dependent activation of the MAP kinase pathway in thyroid cancer cells impairs cell cycle regulation increasing Skp2 and decreasing p27 protein

levels. MAP kinase dependent down-regulation of p27 is prevented by use of proteasome inhibitors and the absence of additive effects on p27 accumulation between the MAP kinase inhibitor U0126 and the proteasome inhibitor MG132, further argued for the implication of the proteasome machinery in the MAP kinase dependent regulation of p27 levels. This conclusion is strengthened by the observation that thyroid tumours with high MAP kinase activity showed high p27 degradation rate whereas tumours with low MAP kinase activity presented low p27 degradation rate.

In conclusion, our findings indicate that Skp2 may play an important role for the development of thyroid cancer according to the current opinion indicating that Skp2 is an oncogene (Nakayama et al. 2000).

The data reported here might provide additional insight into the molecular pathogenesis and identify Skp2 as a novel potential therapeutic target for thyroid ATC.

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