$p27^{Kip1}$ is inactivated in human colorectal cancer by cytoplasmic localization associated with activation of Akt/PKB

C. BOTTINI[1], F. PLATINI[1], M. RINALDI[1], M. LEUTNER[2], O. ALABISO[2,3], M. GARAVOGLIA[2,3] and L. TESSITORE[1]

[1]Department of Food, Chemical, Pharmaceutical and Pharmacological Sciences (DISCAFF), University of East Piedmont 'A. Avogadro', Via Bovio 6, 28100, Novara; [2]'Azienda Ospedaliero-Universitaria Maggiore della Carità', Corso Mazzini 18, 28100, Novara; [3]Department of Clinical and Experimental Medicine, University of East Piedmont 'A. Avogadro', Via Solaroli 17, 28100, Novara, Italy

Received February 1, 2008; Accepted June 19, 2008

DOI: 10.3892/ijo_00000130

Abstract. $p27^{Kip1}$ is a nuclear member of the Kip/Cip family of cyclin-dependent kinase inhibitors and is a negative cell cycle regulator that is thought to play a role in tumour suppression. Reduced levels of $p27^{Kip1}$ are frequent in human cancers and these have been associated with poor prognosis. We have analysed $p27^{Kip1}$ expression and intracellular localization in 70 human colorectal cancers by Western blotting and immunohistochemistry and the results related to Akt expression and clinical pathological parameters. $p27^{Kip1}$ protein expression, as evaluated by Western blotting, was absent or reduced in about 63% of colorectal cancers compared with both peritumoral and normal tissue. Cytoplasmic $p27^{Kip1}$ was detected, by immunohistochemical analysis, in 30% (21 of 70) of cases indicating that translocation of $p27^{Kip1}$ protein into the cytoplasm may be responsible for $p27^{Kip1}$ inactivation. The analysis of phosphorylated Akt by Western blotting indicated that it was expressed in 38% (8 of 21) of tumours showing cytoplasmic $p27^{Kip1}$. Patients whose cancer presented accumulation of cytoplasmic $p27^{Kip1}$ showed poorer outcomes for cancer-related relapse and survival. These results suggest that cytoplasmic $p27^{Kip1}$ localization, associated or not with Akt activation, may contribute to colorectal tumorigenesis and metastasis and it may be useful as a negative prognostic factor for the outcome of patients with colorectal cancer.

Introduction

Most of human cancers show alterations in the expression of genes that regulate cell cycle progression. In mammalian cells, the progression through the cell cycle is controlled by the cyclin-dependent kinases (CDKs) which in turn are regulated by phosphorylation and two groups of proteins, the cyclins and cyclin-dependent kinase inhibitors (CDKIs) (1). There are two families of CDKIs, the Cip/Kip and INK4 families. Members of the Cip/Kip family inhibit the activity of all CDKs and, as a consequence, they are considered as universal inhibitors. In contrast, the members of the INK4 family specifically inhibit the kinase activity of CDK4/CDK6 (2). CDKIs oppose mitogenic stimuli and cause G1 arrest when overexpressed in transfected cells indicating a negative regulatory role for these proteins in tumour suppression (3). $p27^{Kip1}$ was originally identified as an inhibitor of CDK activity in epithelial cells arrested by transforming growth factor (TGFß) (4). $p27^{Kip1}$ is found in the nucleus of cells and its levels are decreased by mitogenic growth factor signalling and are increased in response to signals of differentiation and cell death (5). Levels of $p27^{Kip1}$ are therefore high when cells are in the resting phase and they rapidly decrease after mitosis is stimulated (6,7). Overexpression of $p27^{Kip1}$ leads to a block in G1-S phase transition and induces apoptosis and autophagy (8,9). Mice lacking $p27^{Kip1}$ show increased body size, organomegaly and spontaneous pituitary tumour formation, suggesting that $p27^{Kip1}$ plays an important role in repressing tumour development (10). Consistently, $p27^{Kip1}$ expression is decreased or virtually absent in most common human tumours, including colon cancer (11-16). Nevertheless, deletion, rearrangement and mutation of the $p27^{Kip1}$ gene are rare events in human cancer (6). We have previously reported the downregulation of $p27^{Kip1}$ in the early preneoplastic lesions in a rat model of colorectal cancer (17). Akt has been found to downregulate $p27^{Kip1}$ transcription by phosphorylation dependent inhibition of the Forkhead family of transcription factors (18). However, $p27^{Kip1}$ expression is virtually absent in up to 50% of human tumours largely due to enhanced proteolysis (12,16,19). Paradoxically, some cancers have high levels of $p27^{Kip1}$ protein but the protein is translocated to the cytoplasm (12,16,20). Akt-induced $p27^{Kip1}$ phosphorylation causes retention of $p27^{Kip1}$ protein in the cytoplasm which has been proposed to be a novel mechanism whereby $p27^{Kip1}$ is inactivated in breast cancer (21-23).

The aim of the present study was to investigate whether $p27^{Kip1}$ inactivation in colorectal cancer may be related to

Correspondence to: Professor Luciana Tessitore, Dipartimento DISCAFF, Via Bovio 6, 28100 Novara, Italy
E-mail: tessitore@pharm.unipmn.it

Key words: $p27^{Kip1}$, Akt/PKB, colorectal cancer, cytoplasmic $p27^{Kip1}$

Table I. Clinicopathological features in patients with colorectal cancer.

	Patients
Median age (years)	71
Gender (M/F)	30/40
Tumour site	
Right colon	22
Left colon	9
Transverse colon	4
Sigma	25
Rectal	10
Differentiation degree	
G1	10
G2	47
G3	13
Primary tumour	
T1	1
T2	11
T3	30
T4	28
Regional lymph nodes	
N0	24
N1	23
N2	23
Distant metastasis	
Mx	9
M0	36
M1	25

cytoplasmic translocation associated with Akt activation and poor patient prognosis.

Materials and methods

Patient population. Seventy patients with colorectal cancer were included in this investigation. The population studied underwent surgery for colorectal cancer between 2000 and 2005 at the Maggiore Hospital in Novara. There were 30 men and 40 women patients, ranging in age from 40 to 94 years, with a median age of 71 years. Patients showed diverse tumour sites: 22 right colon carcinoma, 9 left colon carcinoma, 25 sigma carcinoma, 10 rectal carcinoma and 4 transverse colon carcinoma.

The surgically removed segment of colon of each patients was divided into three sections: one zone directly involved in the tumour mass, another peritumoral and the last distal from the tumour site (normal tissue). One part was immediately placed in formalin fixative and then embedded in paraffin wax for immunohistochemistry analysis, the other part were snap-frozen in liquid nitrogen immediately after surgery and stored at -80˚C until used for Western blotting analysis.

Patients and tumour data were recorded in a database for follow-up using a unique identifier. Data recorded for all patients included age, sex, tumour site, tumour staging and differentiation degree. The proliferative index was calculated for each patient as MIB-1. Follow-up information included local, regional and distant recurrence as well as survival data (Table I).

Western blot analysis. For Western blotting, 50 mg of each tissue sample was lysed in a buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% TX-100, 1 mM EGTA, 1 mM DTT), protease inhibitor (Complete Mini EDTA free-Roche) and NaF 0.1 M. Following sonication and incubation on ice for 30 min, the supernatant was collected. Protein concentrations were determined with the Bradford assay. The extracts were cleared by centrifugation and supernatants were stored at -80˚C. The lysates were centrifuged and proteins (25 μg) were subjected to electrophoresis on Tris-glycine SDS polyacrylamide gel. After blotting onto nitrocellulose with filter papers (Bio-Rad Laboratories) non-specific binding sites were blocked by 105 incubations and carried out 1 h at room temperature in phosphate-buffered saline (PBS) with 5% milk. The blots were then probed with two different antibodies; p27^{Kip1} (Santa Cruz) and Phospho-Akt (Cell Signaling Technology) 1:500 for 1 h at room temperature. After being washed they were incubated with secondary antibody 1:5000 for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence according to the manufacturer's instructions (Super Signal, Pierce). Jurkat cell lysates were used as a positive control.

Immunohistochemical analysis. Formalin-fixed, paraffin-embedded tumour samples from surgical specimens were used for this study. Sections (3 μm) were obtained and placed in an oven at 60˚C for 20 min. Tissue sections were deparaffinized with xylene and rehydrated in graded alcohol. Microwave heating of tissue sections immersed in EDTA buffer was used for antigen retrieval. Hydrogen peroxide (3%) was then applied to block endogenous peroxidase activity. All slides were then place in phosphate-buffered saline (PBS) and incubated with blocking solution (Antibody diluent, Dako) for 30 min at room temperature. Sections were then incubated for1 h at room temperature with the monoclonal p27^{kip1} antibody (Dako) at a concentration of 1:50 and 1 h at room temperature with MIB-1 antibody (Dako) at a concentration of 1:100. Secondary antibody (Dako, Envision + Single Reagent HRP) was applied for 30 min at room temperature. Finally 3,3-diaminobenzidine tetrahydrochloride (Dako) was used as the chromophore for 10 min and sections were counterstained with hematoxylin.

Statistical analysis. Data were analysed with the Fisher indepedence test. The outcome was considered statistically significant at $p \leq 0.05$.

Results

p27^{Kip1} inactivation is due to decreased protein levels and cytoplasmic localization. A representative Western blotting

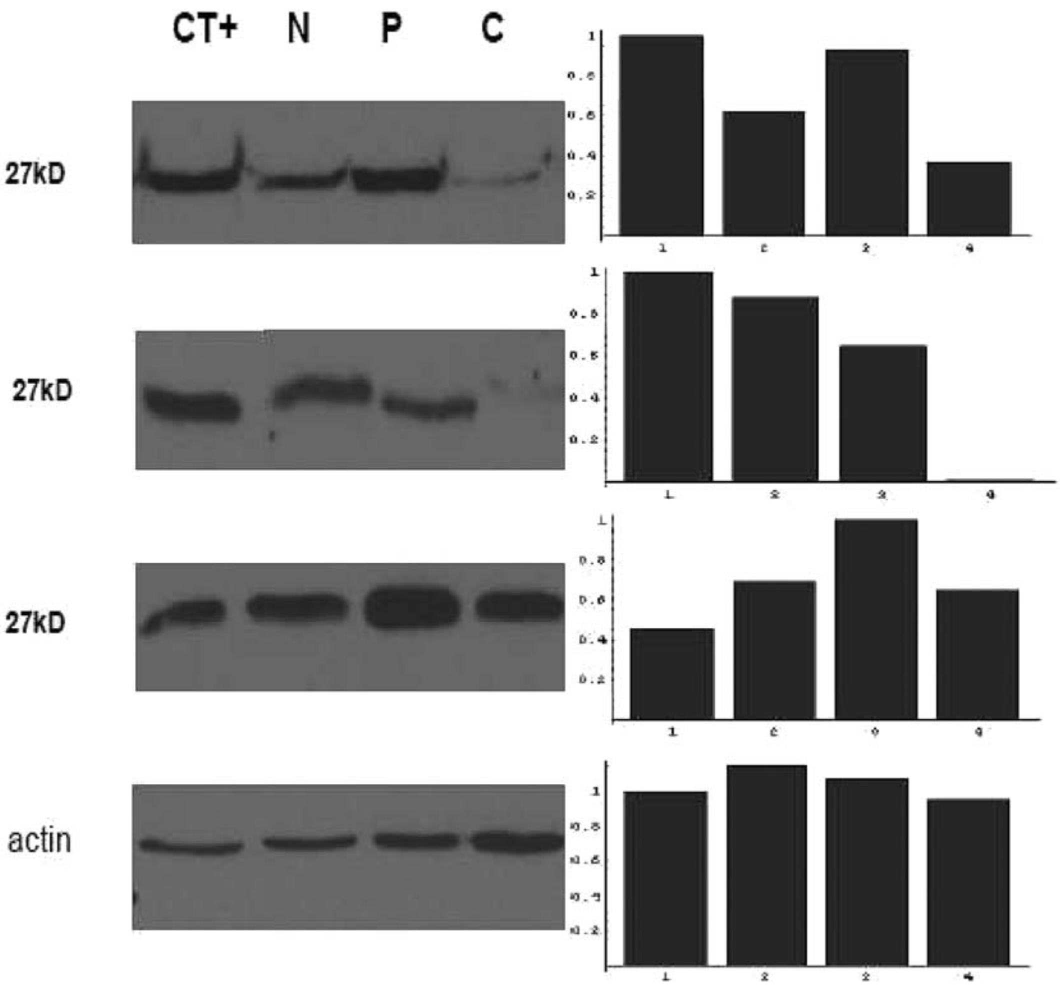

Figure 1. Representative Western blot analysis in human colorectal cancer using anti-p27Kip1. Levels of expression of total p27Kip1 protein in primary colon carcinoma (C) and paired normal adjacent mucosa (P), normal mucosa (N) and Jurkatt cell lines as positive control (CT+). Autoradiographs were subjected to scanning densitometry. The results are shown and expressed as percentage of control values.

Table II. p27 and Akt-P expression in the colorectal cancers.

p27	WB		IHC
		↓10	N 15 → 0 Akt-P
+	36		
		↑26	C 21 → 8 Akt-P
-	34		34
Total	70		70

+, presence of p27; -, absence of p27; N, nuclear localization; C, cytoplasmic localization. p27 was evaluated by Western blotting (WB) and immunohistochemical (IHC) analysis.

of p27Kip1 is shown in Fig. 1. The expression of p27Kip1 was negative in 34/70 (48%) of cancers and positive in 36/70 (52%), when p27Kip1 expression was evident (36/70) it was reduced in the tumour tissue compared to normal tissue and peritumoral tissue in 10/36 (28%) of cancers and surprisingly, 26/36 (72%) cancers showed high levels of p27Kip1 expression similar to those measured in normal tissues (Table II).

To investigate whether cytoplasmic translocation of p27Kip1 might account for the high levels of p27Kip1 protein expression evaluated by Western blotting in 26/70 (37%) tumour tissues, we analyzed the subcellular distribution of p27Kip1 in all the samples of colorectal carcinomas and peritumoral tissue as well as in normal tissue. Immunohistochemical staining for p27Kip1 shows that p27Kip1 protein can be observed in both the nucleus and the cytoplasm (Fig. 2). As described (20), p27Kip1 was scored 'nuclear' when >35% of p27Kip1 expressing cells presented nuclear staining, and 'cytoplasmic' when there was nucleo-cytoplasmic or exclusively cytoplasmic staining in at least 35% of p27Kip1 expressing cells. According to these criteria, positive immunohistochemical staining for the p27Kip1 protein was found in the nucleus of normal colorectal tissue; however, in some samples a few cells showed protein also in the cytoplasm.

The expression of nuclear p27Kip1 levels was always reduced in cancer tissue compared to normal issue. In particular, p27Kip1 expression was not expressed in the same 34 tumour samples out of 70 (48%) which were negative for p27Kip1 at Western blot analysis, in the remaining 36/70 tumour

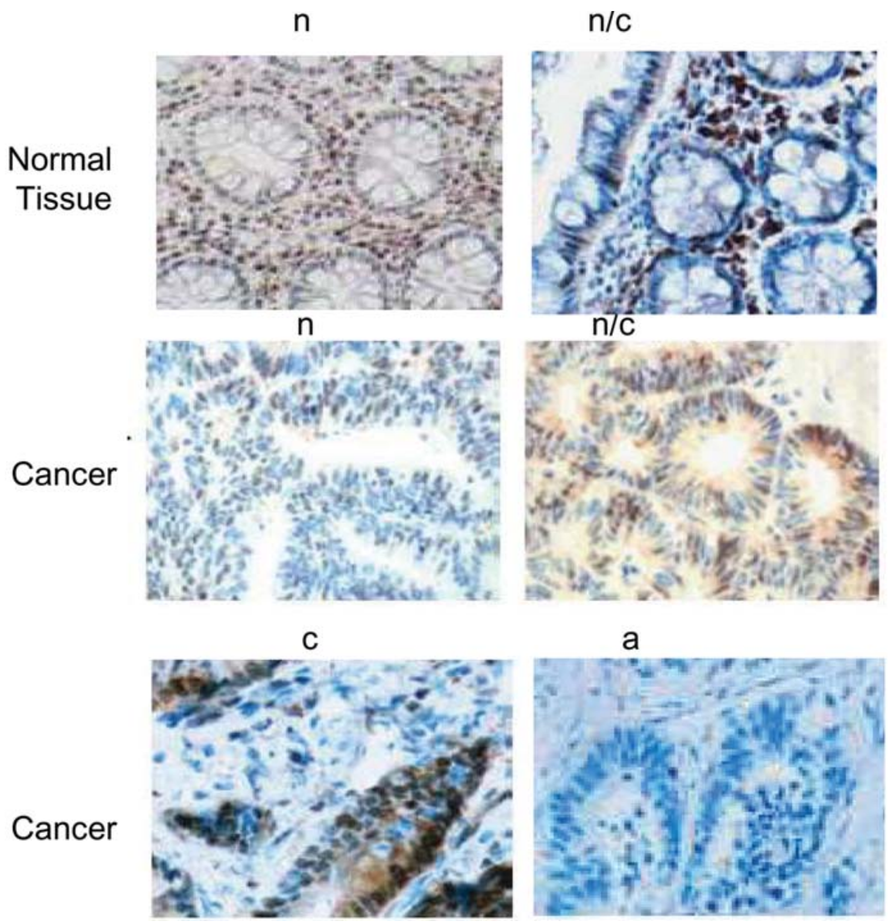

Figure 2. Representative immunohistochemical analysis of p27Kip1 expression in colorectal carcinoma and normal tissue. n, presence of p27Kip1 in the nucleus; n/c, presence of p27Kip1 both in the nucleus and in the cytoplasm; a, absence of p27Kip1. Original magnification x40.

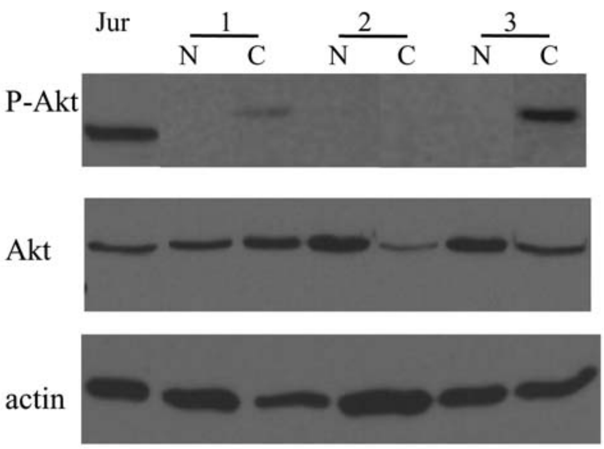

Figure 3. Representative Western blot analysis of patients with colorectal cancer. Three representative samples shown Akt and p-Akt proteins expression in the normal tissue (N) and colorectal cancer (C) of the same patient.

Table III. Relationship between p27 expression and clinical data.

	Patients	p27+	%
Gender			
F	40	22	55
M	30	14	47
Age			
<50	3	1	33
50-60	9	3	33
60-70	24	15	63
>70	34	17	50
Tumour site			
Right colon	22	9	41
Left colon	9	7	78
Transverse colon	4	2	50
Sigma	10	6	60
Rectal	25	12	50

it was nuclear in 15 tumour samples out of 70 (21%), p27Kip1 was expressed in cytoplasm in 21/70 (30%) tumour samples (Table II).

Akt/PKB phosphorylation enhances in some carcinomas the cytoplasmic mislocalization of p27Kip1. To clarify whether activation of PKB/Akt might represent a mechanism for p27Kip1 accumulation in the cytoplasm we evaluated the

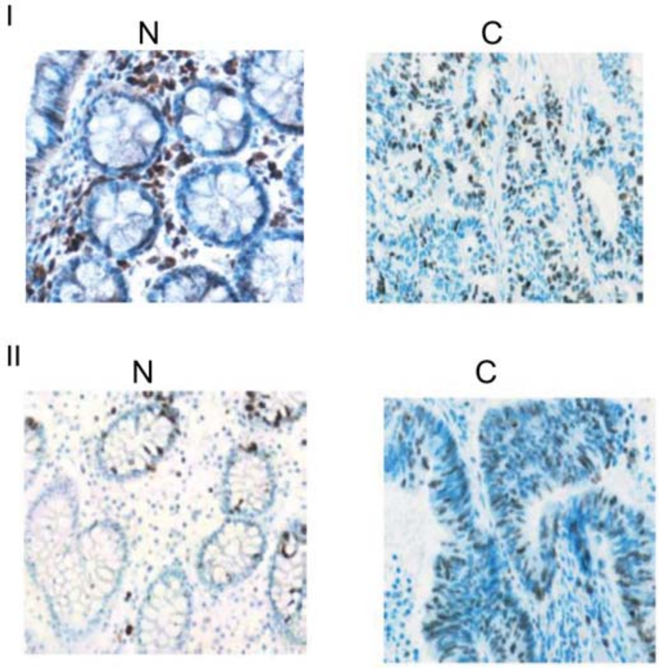

Figure 4. Representative immunohistochemical analysis of the expression of Ki-67 measured with MIB-1 antibody in normal tissue (N) and colorectal cancer (C) of two patients. Original magnification x40.

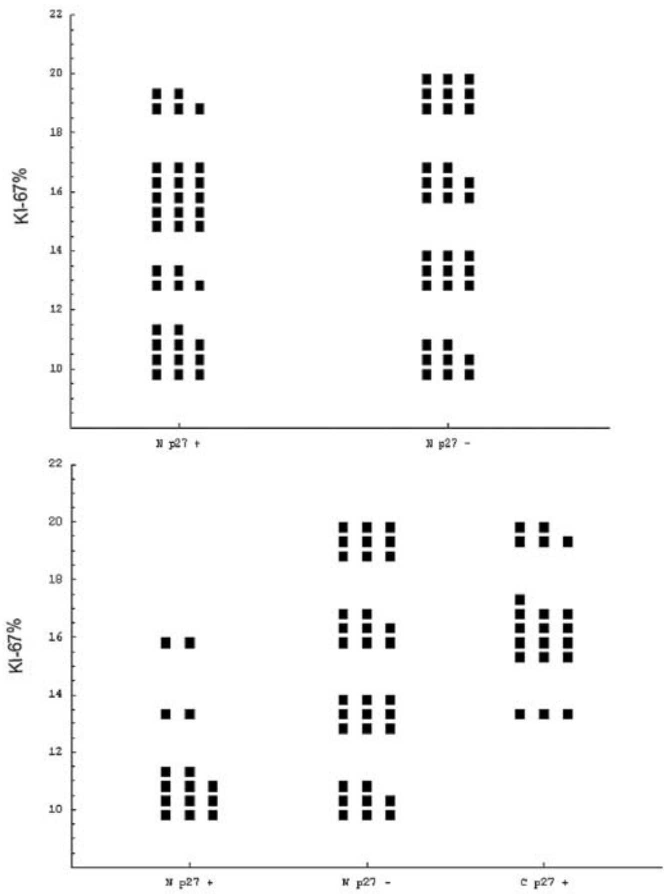

Figure 5. Correlation between tumour proliferative activity and p27Kip1 expression/localization. The proliferative activity was evaluated by MIB-1.

levels of expression of both non-phosphorylated and phosphorylated form of Akt in all 70 tumour samples. We found that the non-phosphorylated form of Akt was expressed at high levels in all 70 neoplastic and non-neoplastic tissue samples, whereas the phosphorylated form of Akt was evident in 8 (38%) out of 21 cancer tissues showing cytoplasmic p27Kip1 as assessed by immunohistochemical analysis (Fig. 3, Table II).

The expression and the localization of p27Kip1 are related to clinicopathological parameters of colorectal carcinomas. The clinicopathological features in terms of patient gender, age and tumour site were similar in cancer subsets differing in p27Kip1 protein expression ($p \geq 0.05$) (Table III). Conversely, p27Kip1 localization was significantly ($p \leq 0.05$) and inversely related with proliferative activity evaluated as MIB-1 (Ki-67%); in particular, the most rapidly proliferating tumours (14/70) showed no p27Kip1 expression in 9/14 (64%) whereas in 5/14 (36%) p27Kip1 positive protein was localized in the cytoplasm as indicated by the immunohistochemical analysis (Figs. 4 and 5 and Table IV). Table IV also shows that 6/13 (46%) differentiation grade III tumours do not express p27Kip1 as evaluated by both Western blotting and immunohistochemistry, while the other 7/13 (54%) p27Kip1 positive tumours at Western blot analysis show the protein in the cytoplasm.

Interestingly, p27 was virtually absent in 19/28 (68%) T4 tumours. p27Kip1 protein, as investigated by immunohistochemistry, was evident in the nucleus of 4/9 (44%) Western blotting p27Kip1 positive tumours and in the cytoplasm of 5/9 (56%) of T4 tumours. The relationship between p27Kip1 expression and lymph node invasion is reported in Table IV; 8/23 (35%) N2 tumours were p27Kip1 positive at Western blot analysis; 3 of them (38%) expressed p27Kip1 in the nucleus and the other 5 (62%) in the cytoplasm. Strong relationship ($p \leq 0.05$) was evident for the metastatic invasion M1 since 8/25 (32%) p27Kip1 positive tumour at Western blot analysis showed the protein localized only in the cytoplasm 8/8 (100%). Correlation between p27Kip1 expression/localization and pathological features shows a significant relationship ($p \leq 0.05$) between the expression of p27Kip1, evaluated by Western blot analysis, and tumour extention, differentiation degree, regional lymph node and distant metastases (Table IV). High relationship ($p \leq 0.05$) is also shown between p27Kip1 localization by immunohistochemical analysis and proliferative index, differentiation degree and distant metastases (Table IV).

p27Kip1 expression is highly related to clinical outcome. Follow-up information was available for 46 patients (median follow-up 32 months; range 8-64 months). Twenty patients out of 46 showed recurrence within 3 years after surgery. When p27Kip1 protein levels were compared with recurrence high significant relationships were noted ($p \leq 0.05$). In particular, Western blot analysis shows that 16 out of 24 tumours with negative expression relapsed in comparison with 5 tumours out of 22 with positive p27Kip1 expression (Table V). All 24 tumours with negative p27Kip1 expression at Western blotting were negative also at immunohistochemical analysis, so that 16 out of 24 relapsed. As far as

Table IVa. Relationship between p27 expression/localization and pathological features.

	p27+ IHC - WB N (%)		p27+ IHC - WB C (%)		p27- IHC - WB	(%)
Ki-67						
Low	11	(58)	0	(0)	8	(42)
Moderate	2	(14)	3	(22)	9	(64)
High	2	(9)	13	(56)	8	(35)
Very high	0	(0)	5	(36)	9	(64)
G						
I	1	(10)	0	(0)	9	(90)
II	14	(30)	14	(30)	19	(41)
III	0	(0	7	(54)	6	(46)
T						
T1	0	(0)	1	(100)	0	(0)
T2	4	(36)	5	(46)	2	(18)
T3	7	(23)	10	(34	13	(43)
T4	4	(14)	5	(18)	19	(68)
N						
N0	7	(29)	10	(42)	7	(29)
N1	5	(22)	6	(26)	12	(52)
N2	3	(13)	5	(22)	15	(65)
M						
Mx	4	(44)	1	(11)	4	(45)
M0	11	(30)	12	(34)	13	(36)
M1	0	(0)	8	(32)	17	(68

Table IVb. Significance between p27 expression/localization and pathological features.

	p WB	p IHC
Ki-67	0.18	0.00002
T	0.02	0.089
G	0.01	0.005
N	0.04	0.17
M	0.04	0.009

p27 expression was evaluated in colorectal cancers by Western blotting (WB) and immunohistochemical (IHC) analysis and p27 expression was related with pathological feautures of tumour such as the proliferative index (Ki-67), the differentiation degree (G), the tumour extension (T), the regional lymph nodes (N) and distant metastases (M).

Table Va. Relationship between p27 expression and survival or relapse.

p27		Survival no.	Survival (%)	Relapse no.	Relapse (%)
11	N	11	(100)	1	(9)
11	C	5	(45)	4	(36)
24	A	8	(33)	16	(67)
46		24		21	

Follow-up information was available for 46 patients. p27 expression was evaluated by immunohistochemical analysis. N, nuclear localization; C, cytoplasmic localization; A, absent expression.

Table Vb. Significance between p27 expression and survival or relapse.

	p-value
Survival	0.0005
Relapse	0.005

Follow-up information was available for 46 patients. p27 expression was evaluated by immunohistochemical analysis.

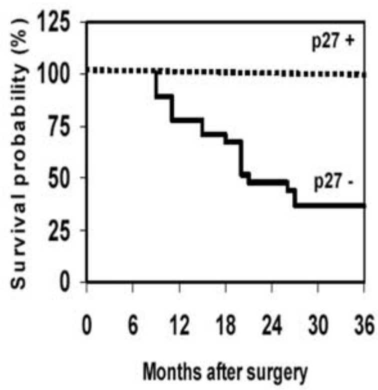

Figure 6. Kaplan-Meier overall survival curve stratified by p27Kip1-positive tumour vs. p27Kip1- negative tumour.

22 tumours showing positive p27Kip1 expression at Western blotting is concerned, 11 tumours showed p27Kip1 in the cytoplasm and 11 in the nucleus in immunohistochemical analysis, 4/11 tumours with cytoplasmic p27Kip1 and only 1/11 tumours with nuclear p27Kip1 showed relapse. On the whole, 20 out of 21 tumours showing recurrence had inactive p27Kip1 expression being either absent (16/21) or cytoplasmic (4/21). When patients with tumours with positive p27Kip1 expression were compared with those with negative expression, a shortened overall survival was seen in those with reduced p27Kip1 levels (Fig. 6). All patients with tumours showing nuclear p27Kip1 levels had a 100% (11/11) survival within 3 years, while only 37% of patients with tumours showing p27Kip1 negative levels (8/24) or with cytoplasmic localization of p27Kip1 (5/11) survived 3 years.

Discussion

In this study, we have presented the first evidence of the prognostic value of cytoplasmic p27Kip1 expression associated with Akt activation in colorectal cancers. A number of studies have shown that low protein level of $p27^{Kip1}$, largely due to enhanced $p27^{Kip1}$ proteolysis (12), is a negative prognostic indicator in various human cancers (24-27), including colorectal cancer (12,28). However, various tumours do express $p27^{Kip1}$, suggesting that other mechanisms must be involved in $p27^{Kip1}$ inactivation in human cancers (29). High levels of $p27^{Kip1}$ expression accompanied by overexpression of cyclin D1 and loss of PTEN protein were seen in human breast cancer in both a number of highly proliferative cell lines *in vitro* as well as in a large number of tumours *in vivo*, suggesting that some tumour cells could overcome $p27^{Kip1}$ inhibition by overexpressing cyclin D1/cdk4 (29,30). Alternatively, a subcellular redistribution of $p27^{Kip1}$ has been proposed to control its function as a negative regulator of cell proliferation in various tumours (21-23,31). The balance between the active nuclear $p27^{Kip1}$ and the inactive cytoplasmic protein appears to be more relevant than the total intracellular level of $p27^{Kip1}$ expression. A novel pathway involving receptor tyrosine kinase signalling has been proposed to mediate either $p27^{Kip1}$ proteolysis or cytoplasmic accumulation in some breast cancers while in others, both occurred (32,33).

Here we have shown that $p27^{Kip1}$ protein was increased in the tumour cell cytoplasm associated with low or absent $p27^{Kip1}$ nuclear levels. Cytoplasmic $p27^{Kip1}$ accumulation may correspond to either neo-synthesized protein or protein translocated from the nucleus to the cytoplasm for degradation. Normally, the neo-synthesized $p27^{Kip1}$ has to be phosphorylated at Thr157 or at Thr187 and Ser10 by activated Akt (23,34). High $p27^{Kip1}$ levels cytoplasmic might be due to Akt activation by PDK1- and PDK2-dependent phosphorylation in Thr308 and Ser473 (35-37). Interestingly, PTEN is frequently inactivated in many types of tumours, including colorectal cancer, where somatic mutations or reduced expression lead to a constitutive activation of Akt that favors colorectal progression (38). $p27^{Kip1}$ phosphorylated at Thr157 is ready for nuclear import dependent on the nuclear localization signal situated near the COOH terminal (34), so importin $\alpha3$ and $\alpha5$ can transport it into the nucleus in conjunction with importin ß (39) and nuclear pore-associated protein-60 (40). Alternatively, $p27^{Kip1}$ phosphorylated at Thr157 can be sequestered by 14-3-3 proteins (34). $p27^{Kip1}$ phosphorylized at Thr187 and Ser10 can either be degraded by SCF-Ubiquitin complex or phosphorylated in a novel Akt-dependent phosphorylation site, the COOH-terminal Thr198 residue to be sequestered by 14-3-3 proteins (9,41).

In the nucleus, $p27^{Kip1}$ can act as a tumour suppressor as indicated by high levels of nuclear $p27^{Kip1}$ expression in quiescent cells and non-proliferative compartments of tissues such as skeletal muscle, cartilage, smooth muscle and fibroblasts. Conversely, low levels are found in highly proliferating neoplastic and non-neoplastic tissues (29). Moreover, overexpression of $p27^{Kip1}$ by transfection in various tumour cell lines was able to reverse the neoplastic phenotype (42-44). It is accepted that inactivation of nuclear $p27^{Kip1}$ occurs when either cyclin E-CDK2 or p-Akt phosphorylated $p27^{Kip1}$ at Thr187, being the first step necessary but insufficient to trigger $p27^{Kip1}$ proteolysis. Subsequently, Thr187 $p27^{Kip1}$ can bind JAB1 in the nucleus and allow export $p27^{Kip1}$ into the cytoplasm. Nuclear activated Akt is able to bind $p27^{Kip1}$ phosphorylized at Thr187 for its further phosphorylation at Ser10 (45). Other nuclear kinases, such as hKIS, are able to phosphorylate $p27^{Kip1}$ at Ser10. In the nucleus of quiescent cells, $p27^{Kip1}$ phosphorylated at both Thr187 and Ser10 inhibits AP-1-dependent immediate early gene expression through repression of JAB1 transcription activity (46). $p27^{Kip1}$ has to be phosphorylated at both Thr187 and Ser10 to increase CRM1/Ran GTP-mediated nuclear export of $p27^{Kip1}$, necessary for cytoplasmic $p27^{Kip1}$ proteasome-dependent proteolysis in early G1 (47). Akt phosphorylates several proteins involved in gene expression for mitogenic and antimitogenic signal transduction and apoptosis (31), contributing to cell cycle regulation, thus impairment of the Akt pathway may be relevant to human carcinogenesis. In this regard, it must be considered that constitutive Akt activation in some colon cancers theoretically might phosphorylate $p27^{Kip1}$, thus favouring the accumulation of inactive cytoplasmic $p27^{Kip1}$. We found an increase in the phosphorylation of Akt in about 38% of tumours showing cytoplasmic $p27^{Kip1}$ in agreement with previous reports on breast cancer (21-23). The enhanced phosphorylation of Akt in colon carcinoma is unlikely to be responsible for reduced $p27^{Kip1}$ transcription as reported in some types of cancer (18), since we found that p-Akt was virtually absent in all the tumours showing low or absent $p27^{Kip1}$ expression.

On the other hand, $p27^{Kip1}$ has been found to be inactivated by enhanced proteolysis via an SCF ubiquitin complex in most colorectal cancers (12) controlled activated Akt (39), suggesting that the accumulation of cytoplasmic $p27^{Kip1}$ was likely to be dependent on $p27^{Kip1}$ sequestration by 14-3-3 proteins rather than reduced protein degradation. Another possibility to be taken into account is the nuclear phosphorylation of $p27^{Kip1}$ at Ser10 by a growth factor activated kinase with subsequent $p27^{Kip1}$ export to the cytoplasm and its novel function in cell motility, independent of its role in cell cycle inhibition (48).

Our results show that $p27^{Kip1}$ inactivation by both reduced nuclear protein levels and accumulation of $p27^{Kip1}$ in the cytoplasm was significantly ($p \leq 0.05$) associated with pathological features indicative of tumour progression such as III differentiation degree, distant metastasis and rapid proliferation. Consistent with the last report, it is worth noting that Fredersdorf *et al* (30) reported an inverse correlation between the expression of $p27^{Kip1}$ and the degree of tumour malignancy in human colorectal tumours. The most severe prognosis in terms of local recurrence and death for our patients is high significantly ($p \leq 0.05$) associated with virtually absent $p27^{Kip1}$ expression in both nucleus and cytoplasm; however, a clear trend towards a worse prognosis for $p27^{Kip1}$ nuclear protein-negative tumour associated with $p27^{Kip1}$ cytoplasmic protein-positive tumours emerged in comparison with $p27^{Kip1}$ nuclear protein-positive cases. Our data are consistent with previous observations in patients with Barrett's associated adenocarcinoma and breast cancer. Besides low $p27^{Kip1}$ protein, also cytoplasmic localization of $p27^{Kip1}$ was associated with unfavourable outcome in Barrett's associated adenocarcinoma (30). Liang *et al* (23) reported that cytoplasmic translocation of $p27^{Kip1}$ worsened the prognosis associated with reduced $p27^{Kip1}$ levels in breast cancer, supporting the relevance of these mechanisms to human carcinogenesis. Interestingly, high levels of $p27^{Kip1}$ expression have been found to correlate with lymph node status in a subset of advanced invasive breast carcinomas, suggesting a role for $p27^{Kip1}$ in cell migration (49).

Taken together, these observations suggest a role for cytoplasmic $p27^{Kip1}$ associated with Akt activation in cell cycle arrest and cell migration leading to poor prognosis for patients with colorectal cancer.

Acknowledgments

The authors thank Mr Richard Billington for correcting the English language and Jan Willem Van de Loo for technical assistance. This work was supported by grants from Regione Piemonte, Lega Italiana Contro i Tumori of Novara, Fondazione CRT and University of East Piedmont 'Amedeo Avogadro'.

References

1. Morgan DO: Principles of Cdk regulation. Nature 374: 131-134, 1995.
2. Lloyd RV, Erickson LA, Jin L, *et al*: P27kip1: a multifunctional dependent kinase. Am J Pathol 154: 313-323, 1999.
3. Macaluso M, Montanari M, Cinti C and Giordano A: Modulation of cell cycle components by epigenetic and genetic events. Semin Oncol 32: 452-457, 2005.
4. Slingerland JM, Hengst L, Pan CH, Alexander D, Stampfen MR and Reed SI: A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor ß-arrested epithelial cells. Mol Cell Biol 14: 3683-3694, 1994.
5. Wang QM, Jones JB and Studzinski GP: Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 Cells. Cancer Res 56: 264-267, 1996.
6. Nourse J, Firpo E, Flanagan WM, *et al*: Interleukin-2-mediated elimination of the p27kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. Nature 372: 570-573, 1994.
7. Coats S, Flanagan WM, Nourse J and Roberts JM: Requirement of p27kip1 for restriction point control of the fibroblast cell cycle. Science 272: 877-880, 1996.
8. Russo AA, Jeffrey PD, Patten AK, Massague J and Pavletich NP: Crystal structure of the p27kip1 cyclin-dependent-kinase inhibitor bound to cyclin A-Cdk complex. Nature 382: 325-331, 1996.
9. Liang J, Shao SH, Xu ZH, *et al*: The energy sensing LKB1-AMPK pathway regulates p27Kip1 phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol 9: 218-224, 2007.
10. Nakayama K, Ishida N, Shirane M, *et al*: Mice lacking p27 (Kip1) display increased body size, multiple organ hyperplasia, retinal displasia and pituitary tumours. Cell 85: 707-720, 1996.
11. Esposito V, Baldi A, De Luca A, *et al*: Prognostic role of the cyclin-dependent kinase in non-small cell lung cancer. Cancer Res 57: 3381-3385, 1997.
12. Loda M, Cukor B, Tam SW, *et al*: Increased proteosome dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. Nat Med 3: 231-234, 1997.
13. Mineta H, Miura K, Suzuki I, *et al*: Low p27 expression correlates with poor prognosis for patients with oral tongue squamous cell carcinoma. Cancer 85: 1011-1017, 1995.
14. Kuczyk M, Machtens S, Hradil K, *et al*: Predictive value of decreased p27 kip1 protein expression for the recurrence-free and long-term survival of prostate cancer patients. Br J Cancer 81: 1052-1058, 1999.
15. Kamai T, Takagi K, Asami H, Ito Y, Oshima H and Yoshida KI: Decreasing of p27(Kip1) and cyclin E protein levels is associated with progression from superficial into bladder cancer. Br J Cancer 84: 1242-1251, 2001.
16. Catzavelos C, Bhattacharya N, Ung YC, *et al*: Decreased levels of the cell-cycle inhibitor p27Kip1 protein: prognostic implication in primary breast cancer. Nat Med 3: 227-230, 1997.
17. Femia AP, Caderni G, Bottini C, Salvadori M, Dolara P and Tessitore L: Mucin-depleted foci are modulated by dietary treatments and show deregulation of proliferative activity in carcinogen-treated rodents. Int J Cancer 120: 2301-2305, 2007.
18. Medema RH, Kops GJ, Bos JL and Burgering BM: AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature 404: 782-787, 2000.
19. Lloyd RV, Jin L, Qian X and Kuling E: Aberrant p27kip1 expression in endocrine and other tumours. Am J Pathol 150: 401-407, 1997.
20. Besson A, Gurian-West M, Chen X, Kelly-Spratt KS, Kemp CJ and Roberts JM: A pathway in quiescent cells that controls p27Kip1 stability, subcellular localization, and tumor suppression. Genes Dev 20: 47-64, 2006.
21. Viglietto G, Motti ML, Bruni P, *et al*: Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27kip1 by PKB/Akt mediated phosphorylation in breast cancer. Nat Med 8: 1136-1144, 2002.
22. Shin I, Yakes FM, Shin NY, Bakin AV, Baselga J and Arteaga CL: PKB/Akt mediates cell-cycle progression by phosphorylation of p27kip1 at threonine 157 and modulation of its cellular localization. Nat Med 8: 1145-1152, 2002.
23. Liang J, Zubovitz J, Petrocelli T, *et al*: PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. Nat Med 8: 1153-1160, 2002.
24. Matsunobu T, Tanaka K, Matsumoto Y, Nakatani F and Sakimura R: The prognostic and therapeutic relevance of p27kip1 in Ewing's family tumours. Clin Cancer Res 10: 1003-1012, 2004.
25. Nitti D, Bellucco C, Mammano E, Marchet A, Amvrosi A and Mencarelli R: Low level of p27kip1 protein expression in gastric adenocarcinoma is associated with disease progression and poor outcome. J Surg Oncol 81: 167-176, 2002.
26. Barnes A, Pinder SE, Bell JA, *et al*: Expression of p27kip1 in breast cancer and its prognostic significance. J Pathol 201: 451-459, 2003.
27. Galizia G, Ferraraccio F, Lieto E, *et al*: p27 downregulation and metallothionein overexpression in gastric cancer patients are associated with a poor survival rate. J Surg Oncol 93: 241-252, 2006.
28. Gunther K, Jung A, Volker U, *et al*: p27(kip1) expression in rectal cancer correlates with disease-free survival. J Surg Res 92: 78-84, 2000.
29. Engin H, Baltali E, Guler N, Guler G, Tekuzman G and Uner A: Expression of PTEN, cyclin D1, P27/KIP1 in invasive ductal carcinomas of the breast and correlation with clinicopathological parameters. Bull Cancer 93: E21-E26, 2006.
30. Fredersdorf S, Burns J, Milene AM, *et al*: High level expression of p27kip1 and cyclin D1 in some human breast cancer cells: inverse correlation between the expression of p27kip1 and degree of malignancy in human breast and colorectal cancers. Proc Natl Acad Sci USA 94: 6380-6385, 1997.
31. Singh SP, Lipman J, Goldman H, *et al*: Loss or altered subcellular localization of p27kip1 in Barrett's associated adenocarcinoma. Cancer Res 58: 1730-1735, 1998.
32. Gottschalk AR, Doan A, Nakamura JL, Haas-Kogan DA and Stokoe D: Inhibition of phosphatidylinositol-3-kinase causes cell death through a protein kinase B (PKB)-dependent mechanism and growth arrest through a PKB-independent mechanism. Int J Radiat Oncol Biol Phys 61: 1183-1188, 2005.
33. Coffer PJ, Jin J and Woodgett JR: Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem J 335: 1-13,1998.
34. Zeng Y, Hirano K, Hirano M, Nishimura J and Kanaide H: Minimal requirements for the nuclear localization of p27(Kip1), a cyclin-dependent kinase inhibitor. Biochem Biophys Res Commun 274: 37-42, 2000.
35. Hlobilkova A, Knillova J, Svachova M, Skypalova P, Krystof V and Kolar Z: Tumour suppressor PTEN regulates cell cycle and protein kinase B/Akt pathway in breast cancer cells. Anticancer Res 26: 1015-1022, 2006.
36. Delcommenne M, Tan C, Gray V and Rue L: Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc Natl Acad Sci 95: 11211-11216, 1998.
37. Balendran A, Casamayor A and Deak M: PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. Curr Biol 9: 393-404, 1999.

38. Nassif NT, Lobo GP and Wu X: PTEN mutations are common in sporadic microsatellite stable colorectal cancer. Oncogene 23: 617-628, 2004.
39. Catimel B, The T and Fontes MR: Biophysical characterization of interactions involving importin-alpha during nuclear import. J Biol Chem 276: 34189-34198, 2001.
40. Muller D, Thieke K, Burgin A, Dickmanns A and Eilers M: Cyclin E-mediated elimination of p27 requires its interaction with the nuclear pore-associated protein mNPAP60. EMBO J 19: 2168-2180, 2000.
41. Fujita N, Sato S, Katayama K and Tsuruo T: Akt-dependent phosphorylation of p27kip promotes binding to 14-3-3 and cytoplasmic localization. J Biol Chem 9: 28706-28713, 2002.
42. Katayose Y, Kim M, Rakkar AN, Li Z, Cowan KH and Seth P: Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. Cancer Res 57: 5441-5445, 1997.
43. Naruse I, Hoshino H, Dobashi K, Minato K, Saito R and Mori M: Overexpression of p27kip1 induces growth arrest and apoptosis mediated by changes of pRb expression in lung. Int J Cancer 88: 377-383, 2000.
44. Katner AL, Hoanf QBL and Gootam P: Introduction of cell cycle arrest and apoptosis in human prostate carcinoma cells by a recombinant adenovirus expressing p27kip1. Prostate 53: 77-87, 2002.
45. Ishida N, Hara T, Kamura T, Yoshida M, Nakayama K and Nakayama KI: Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export. J Biol Chem 277: 14355-14358, 2002.
46. Chopra S, Fernandez DE, Mattos S, Lam EWF and Mann DJ: Jab1 Co-activation of c-Jun is abrogated by Serine 10-phosphorylated form of p27kip1. J Biol Chem 36: 32413-32416, 2002.
47. Connor MK, Kotchetkov R and Cariou S: CRM1/Ran-Mediated nuclear export of p27kip1 involves a nuclear export signal and links p27 export and proteolysis. Mol Biol Cell 1: 201-213, 2003.
48. McAllister SS, Becker-Hapak M, Pintucci G, Pagano M and Dowdy SF: Novel p27kip1 C431 terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions. Mol Cell Biol 23: 216-228, 2003.
49. Kouvaraki M, Gorgoulis VG and Rassidakis GZ: High expression levels of p27 correlate with lymph node status in a subset of advanced invasive breast carcinomas. Cancer 94: 2454-2465, 2002.