Abstract. p27Kip1 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 p27Kip1 are frequent in human cancers and these have been associated with poor prognosis. We have analysed p27Kip1 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. p27Kip1 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 p27Kip1 was detected, by immunohistochemical analysis, in 30% (21 of 70) of cases indicating that translocation of p27Kip1 protein into the cytoplasm may be responsible for p27Kip1 inactivation. The analysis of phosphorylated Akt by Western blotting indicated that it was expressed in 38% (8 of 21) of tumours showing cytoplasmic p27Kip1. Patients whose cancer presented accumulation of cytoplasmic p27Kip1 showed poorer outcomes for cancer-related relapse and survival. These results suggest that cytoplasmic p27Kip1 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). p27Kip1 was originally identified as an inhibitor of CDK activity in epithelial cells arrested by transforming growth factor (TGFß) (4). p27Kip1 is found in the nucleus of cells and its levels are decreased in response to signals of differentiation and cell death (5). Levels of p27Kip1 are therefore high when cells are in the resting phase and they rapidly decrease after mitosis is stimulated (6,7). Overexpression of p27Kip1 leads to a block in G1-S phase transition and induces apoptosis and autophagy (8,9). Mice lacking p27Kip1 show increased body size, organomegaly and spontaneous pituitary tumour formation, suggesting that p27Kip1 plays an important role in repressing tumour development (10). Consistently, p27Kip1 expression is virtually absent in most common human tumours, including colon cancer (11-16). Nevertheless, deletion, rearrangement and mutation of the p27Kip1 gene are rare events in human cancer (6). We have previously reported the downregulation of p27Kip1 in the early preneoplastic lesions in a rat model of colorectal cancer (17). Akt has been found to downregulate p27Kip1 transcription by phosphorylation dependent inhibition of the Forkhead family of transcription factors (18). However, p27Kip1 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 p27Kip1 protein but the protein is translocated to the cytoplasm (12,16,20). Akt-induced p27Kip1 phosphorylation causes retention of p27Kip1 protein in the cytoplasm which has been proposed to be a novel mechanism whereby p27Kip1 is inactivated in breast cancer (21-23).

The aim of the present study was to investigate whether p27Kip1 inactivation in colorectal cancer may be related to...
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 patient 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.

**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 MgCl₂, 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) 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.

**Statistical analysis.** Data were analysed with the Fisher independence test. The outcome was considered statistically significant at p≤0.05.

**Results**

p27Kip1 inactivation is due to decreased protein levels and cytoplasmic localization. A representative Western blotting
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 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 tissue. 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

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

<table>
<thead>
<tr>
<th>p27</th>
<th>WB</th>
<th>IHC</th>
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<tr>
<td>+</td>
<td>36</td>
<td>N 15 - 0 Akt-P</td>
</tr>
<tr>
<td>-</td>
<td>34</td>
<td>C 21 - 8 Akt-P</td>
</tr>
</tbody>
</table>

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.
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

Table III. Relationship between p27 expression and clinical data.

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>p27+</th>
<th>%</th>
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<tbody>
<tr>
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<tr>
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<td>22</td>
<td>55</td>
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<td>M</td>
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<tr>
<td>&lt;50</td>
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<tr>
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<td>3</td>
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<td>60-70</td>
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<tr>
<td>&gt;70</td>
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<td>17</td>
<td>50</td>
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<td>9</td>
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<tr>
<td>Transverse colon</td>
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</tr>
<tr>
<td>Sigma</td>
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<td>6</td>
<td>60</td>
</tr>
<tr>
<td>Rectal</td>
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<td>12</td>
<td>50</td>
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</table>

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.
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 p27\textsuperscript{Kip1} as assessed by immunohistochemical analysis (Fig. 3, Table II).

The expression and the localization of p27\textsuperscript{Kip1} 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 p27\textsuperscript{Kip1} protein expression (p≥0.05) (Table III). Conversely, p27\textsuperscript{Kip1} localization was significantly (p≤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 p27\textsuperscript{Kip1} expression in 9/14 (64%) whereas in 5/14 (36%) p27\textsuperscript{Kip1} 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 p27\textsuperscript{Kip1} as evaluated by both Western blotting and immunohistochemistry, while the other 7/13 (54%) p27\textsuperscript{Kip1} positive tumours was localized in the cytoplasm.

Interestingly, p27 was virtually absent in 19/28 (68%) T4 tumours. p27\textsuperscript{Kip1} protein, as investigated by immunohistochemistry, was evident in the nucleus of 4/9 (44%) Western blotting p27\textsuperscript{Kip1} positive tumours and in the cytoplasm of 5/9 (56%) of T4 tumours. The relationship between p27\textsuperscript{Kip1} expression and lymph node invasion is reported in Table IV; 8/23 (35%) N2 tumours were p27\textsuperscript{Kip1} positive at Western blot analysis; 3 of them (38%) expressed p27\textsuperscript{Kip1} in the nucleus and the other 5 (62%) in the cytoplasm. Strong relationship (p≤0.05) was evident for the metastatic invasion M1 since 8/25 (32%) p27\textsuperscript{Kip1} positive tumour at Western blot analysis showed the protein localized only in the cytoplasm 8/8 (100%). Correlation between p27\textsuperscript{Kip1} expression/localization and pathological features shows a significant relationship (p≤0.05) between the expression of p27\textsuperscript{Kip1}, evaluated by Western blot analysis, and tumour extension, differentiation degree, regional lymph node and distant metastases (Table IV). High relationship (p≤0.05) is also shown between p27\textsuperscript{Kip1} localization by immunohistochemical analysis and proliferative index, differentiation degree and distant metastases (Table IV).

p27\textsuperscript{Kip1} 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 p27\textsuperscript{Kip1} protein levels were compared with recurrence high significant relationships were noted (p≤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 p27\textsuperscript{Kip1} expression (Table V). All 24 tumours with negative p27\textsuperscript{Kip1} expression at Western blotting were negative also at immunohistochemical analysis, so that 16 out of 24 relapsed. As far as
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 p27Kip1, largely due to enhanced p27Kip1 proteolysis (12), is a negative prognostic indicator in various human cancers (24-27), including colorectal cancer (12,28). However, various tumours do express p27Kip1, suggesting that other mechanisms must be involved in p27Kip1 inactivation in human cancers (29). High levels of p27Kip1 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 p27Kip1 inhibition by overexpressing cyclin D1/cdk4 (29,30). Alternatively, a subcellular redistribution of p27Kip1 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 p27Kip1 and the inactive cytoplasmic protein appears to be more relevant than the total intracellular level of p27Kip1 expression. A novel pathway involving receptor tyrosine kinase signalling has been proposed to mediate either p27Kip1 proteolysis or cytoplasmic accumulation in some breast cancers while in others, both occurred (32,33).

Here we have shown that p27Kip1 protein was increased in the tumour cell cytoplasm associated with low or absent p27Kip1 nuclear levels. Cytoplasmic p27Kip1 accumulation may correspond to either neo-synthesized protein or protein translocated from the nucleus to the cytoplasm for degradation. Normally, the neo-synthesized p27Kip1 has to be phosphorylated at Thr157 or at Thr187 and Ser10 by activated Akt (23,34). High p27Kip1 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). p27Kip1 phosphorylated at Thr157 is ready for nuclear import dependent on the nuclear localization signal situated near the COOH terminal (34), so importin α3 and α5 can transport it into the nucleus in conjunction with importin β (39) and nuclear pore-associated protein-60 (40). Alternatively, p27Kip1 phosphorylated at Thr157 can be sequestered by 14-3-3 proteins (34). p27Kip1 phosphorylated 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, p27Kip1 can act as a tumour suppressor as indicated by high levels of nuclear p27Kip1 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 p27Kip1 by transfection in various tumour cell lines was able to reverse the neoplastic phenotype (42-44). It is accepted that inactivation of nuclear p27Kip1 occurs when either cyclin E-CDK2 or p-Akt phosphorylated p27Kip1 at Thr187, being the first step necessary but insufficient to trigger p27Kip1 proteolysis. Subsequently, Thr187 p27Kip1 can bind JAB1 in the nucleus and allow export p27Kip1 into the cytoplasm. Nuclear activated Akt is able to bind p27Kip1 phosphorylated at Thr187 for its further phosphorylation at Ser10 (45). Other nuclear kinases, such as hKIS, are able to phosphorylate p27Kip1 at Ser10. In the nucleus of quiescent cells, p27Kip1 phosphorylated at both Thr187 and Ser10 inhibits AP-1-dependent immediate early gene expression through repression of JAB1 transcription activity (46). p27Kip1 has to be phosphorylated at both Thr187 and Ser10 to increase CRM1/Ran GTP-mediated nuclear export of p27Kip1, necessary for cytoplasmic p27Kip1 proteosome-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 p27Kip1, thus favouring the accumulation of inactive cytoplasmic p27Kip1.

We found an increase in the phosphorylation of Akt in about 38% of tumours showing cytoplasmic p27Kip1 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 p27Kip1 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 p27Kip1 expression.

On the other hand, p27Kip1 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 p27Kip1 was likely to be dependent on p27Kip1 sequestration by 14-3-3 proteins rather than reduced protein degradation. Another possibility to be taken into account is the nuclear phosphorylation of p27Kip1 at Ser10 by a growth factor activated kinase with subsequent p27Kip1 export to the cytoplasm and its novel function in cell motility, independent of its role in cell cycle inhibition (48).

Our results show that p27Kip1 inactivation by both reduced nuclear protein levels and accumulation of p27Kip1 in the cytoplasm was significantly (p≤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 p27Kip1 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≤0.05) associated with virtually absent p27Kip1 expression in both nucleus and cytoplasm; however, a clear trend towards a worse prognosis for p27Kip1 nuclear protein-negative tumour associated with p27Kip1 cytoplasmic protein-positive tumours emerged in comparison with p27Kip1 nuclear protein-positive cases. Our data are consistent with previous observations in patients with Barrett’s associated adenocarcinoma and breast cancer. Besides low p27Kip1 protein, also cytoplasmic localization of p27Kip1 was associated with unfavourable outcome in Barrett’s associated adenocarcinoma (30). Liang et al (23) reported that cytoplasmic translocation of p27Kip1 worsened the prognosis associated with reduced p27Kip1 levels in breast
cancer, supporting the relevance of these mechanisms to human carcinogenesis. Interestingly, high levels of p27Kip1 expression have been found to correlate with lymph node status in a subset of advanced invasive breast carcinomas, suggesting a role for p27Kip1 in cell migration (49).

Taken together, these observations suggest a role for cytoplasmic p27Kip1 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’.

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