Prognostic Role of the Cyclin-dependent Kinase Inhibitor p27 in Non-Small Cell Lung Cancer

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Abstract

Despite its potential role as a tumor suppressor, p27 gene, a member of the Cip/Kip family of cyclin-dependent kinase inhibitor genes, has never been found mutated in human tumors. We investigated p27 protein expression in a series of 108 non-small cell lung cancers (57.4% stage 1, 16.7% stage 2, and 25.9% stage 3) to determine whether the lack or altered expression of this protein correlates with neoplastic transformation and/or progression. We performed immunohistochemistry and Western blot analysis of each specimen. We found that tumors expressing low or undetectable levels of p27 contained high p27 degradation activity. When we evaluated the outcome of the patients in relationship to p27 expression, we found p27 to be a prognostic factor correlating with the overall survival times (P = 0.0012).

The possibility of a simple assay, such as the immunohistochemical analysis of p27 expression on routinely formalin-fixed, paraffin-embedded specimens, has considerable value for the prognosis of patients who undergo surgical resection. In addition, confirmation of the involvement of the proteasome-mediated proteolysis in p27 degradation should stimulate new strategies of nonsurgical treatments of non-small cell lung cancer.

Introduction

The eukaryotic cell cycle is controlled by protein complexes composed of cyclins and cdks5 (1). The regulatory function of cdks is achieved by phosphorylation of key substrates, such as the members of the retinoblastoma gene family (2).

The activity of cdks is regulated by posttranslational modification and by the association-dissociation with inhibitory subunit-designated CKIs (1). Two families of these inhibitors have been identified in mammalian cells. Members of each of the two families share a high percentage of sequence homology, in addition to their specificity of interaction with cdks. The first family, which includes p21 (also known as Cip1, Pic1, Sdi1, mdm6, and Waf1), p27 (also known as Ink, Kipl, and Pic2), and p57 (also called Kip2), preferentially inhibits cdk2. The second family, which includes p16 (also known as Ink4A, Mts1, Cdkn2, and Cdkn4A), p15 (also called Ink4B and Mts2), p18 (also called Ink4C and Ink6A), and p19/p20 (also called Ink4D and Ink6B), preferentially binds to and inhibits cdk4 and cdk6.

Results from different lines of investigation have indicated that CKIs are the products of potential tumor suppressor genes (1). p27 was first identified in complexes with cdk2/cyclin E in Transforming growth factor β-arrested cells (3). p27 protein associates with cyclin E/cdk2 and cyclin A/cdk2 complexes and abrogates their activities. It is a negative cell cycle regulator implicated in G1 phase arrest by transforming growth factor β cell contact with inhibition agents that increase the level of cyclic AMP, staurosporin, lovastatin, tamoxifen, and rapamycin. Overexpression of p27 protein in mammalian cells induces a G1 block of the cell cycle (4,5). In addition, high levels of p27 have been found in quiescent cells, thus implying a role for p27 in maintaining cells in G0 (6–8). Levels of p27 decrease as cells reenter the cell cycle, mostly due to ubiquitin-proteasome-dependent degradation (9).

Lung cancer survival in humans has not improved (~13%) over the past two decades, despite continuing research into new therapeutic strategies. Only approximately 30% of patients are eligible for radical lung resection, and only about one-third of these patients are free from disease 5 years after surgery (10). Cell cycle regulator alterations may have prognostic value in identifying those patients most likely to benefit from radical lung resection. No structural alteration of p27 gene has been detected in human neoplasms at this time (11–16). We studied p27 protein expression in tissue samples from 108 patients affected by non-small cell lung cancer and correlated the results with the outcomes of the patients.

Materials and Methods

Population Study. One hundred and eight non-small cell lung cancer specimens were obtained from patients who underwent a surgical resection (lobectomy or pneumonectomy) in the departments of Thoracic Surgery of the V. Monaldi Hospital and of the II University of Naples (Italy) between 1988 and 1992. All specimens were from patients who had not received chemo- or radiotherapy prior to surgical resection. Outcome data were collected from hospital charts and from periodic interviews with patients and their relatives. The follow-up period was 48 months from the date of surgery (for survivors). Patients who died of causes other than lung cancer were not included in the study. The mean age was 60.1. Gender was unevenly distributed, with females accounting for only 7.4% of the population (eight patients).

The histological diagnoses and classifications of the tumors were based on WHO criteria (17). The postsurgical pathologic Tumor-Node-Metastasis stage was determined according to the guidelines of the American Joint Committee on Cancer (18).

After surgical resection, each tumor specimen was divided into two parts. The first part was frozen instantly for the extraction of RNAs and proteins. The
second part was formalin fixed immediately and then paraffin embedded for routine and immunohistochemical investigation.

**Immunohistochemistry.** Immunohistochemistry has been performed as described previously (19). All samples were processed under the same conditions. Two pathologists (A. Baldi and F. Baldi) separately evaluated the staining pattern of the protein and scored it for the percentage of positive nuclei: score 1, less than 5% of positive cells; score 2, from 5 to 50% of positive cells; score 2, more than 50% of positive cells. At least 20 high power fields were chosen randomly, and 2000 cells were counted.

**Western Blot.** One g of each frozen lung cancer tissue sample was sectioned and quickly homogenized at 4°C in 250 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 0.1% (v/v), Trition X-100, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 0.5 mM Na3VO4, 10 mg/ml leupeptin, and 50 mg/ml aprotinin. The homogenates were cleared by centrifugation for 15 min at 13,000 x g at 4°C, and total protein in the extracts was determined. Western blots were performed as described previously (20).

**Northern Blot Analysis.** Northern blots were performed as described previously (20).

**Degradation Assay.** One g of each frozen human tissue sample (12 representative samples) was sectioned and quickly homogenized at 15,000 rpm in 1 ml of ice-cold, doubly distilled water. The sample was frozen and thawed three times. The lysate was spun down at 15,000 rpm for 45 min at 4°C. The supernatant was retrieved and frozen at −80°C. This method of preparation of total extract preserves ubiquitinating enzymes (9).

Purified histidine-tagged p27 (0.06 μl) was incubated at 37°C for different times in 30 μl of degradation mix containing 100 μg of protein human tissue extracts, 50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 1 mM DTT, 2 mM ATP, 10 mM creatine phosphate, and 5 μM ubiquitin. Degradation of p27 was analyzed by immunoblotting with p27 monoclonal antibody. To remove the proteasome, tumor sample were ultracentrifuged at 100,000 x g for 6 h as described previously (9).

**Statistical Analysis.** Patient survival data were used to determine possible correlation between p27 expression levels and overall survival time. Survival curves were constructed using Kaplan-Meier analysis. Statistical significance of these data was measured by the Mantel-Cox test. Possible associations between the different variables of the analyzed tumor samples were tested for association by the χ² test.

**Results**

**p27 Protein Expression in Non-Small Cell Lung Cancer.** Routine histological assays were performed on 108 non-small cell lung cancers and evaluated independently by p27 immunostaining. Sixty-two tumors were found to be adenocarcinomas, and 46 were squamous carcinomas. Sixty-two tumors (57.4%) were classified as stage I, 18 tumors (16.7%) were classified as stage II, and 28 tumors (25.9%) were classified as stage III. Thirty-two tumors were considered low differentiated (29.6%), 42 tumors were medium differentiated (38.9%), and 34 tumors were well differentiated (31.5%).

Immunoreactivity for p27 was found in both normal and neoplastic tissues. p27 immunostaining was always nuclear, with a low to absent background. p27 expression in normal lung tissue was detected in bronchial epithelia (mostly in ciliated cells) and in the adjacent glands (Fig. 1A). Pneumocytes displayed moderate nuclear immunoreactivity. The staining was specific because it was blocked by preincubation of the antibody with recombinant purified p27 but not with an irrelevant protein (data not shown).

p27 immunostaining was found in 96 (88.9%) of the investigated specimens (Fig. 1B). The percentage of positive cells in the remaining 12 specimens (11.1%) ranged from 0 to 5% (Fig. 1C). No statistically significant difference in the p27 expression was found between adenocarcinomas and squamous carcinomas.

We decided to investigate if a comparable p27 expression was found in the same specimens analyzed by Western blot. Protein extracts were prepared from all of the 108 frozen samples. p27 protein was found to be clearly expressed at different levels in the specimens examined. In particular, 12 of the 108 specimens showed undetectable levels of p27 protein expression. Fig. 2A shows a representative panel of p27 with a different expression level. To check that the same amount of protein was present in each lysate and that the samples were not degraded, the same blot was normalized with the monoclonal antibody heat shock protein 72/73 purchased from Oncogene Science (data not shown). Interestingly, these results correlate statistically with the immunohistochemical assays (P < 0.001).

**Low Levels of p27 Protein Are Directly Correlated with High p27 Degradation Activity.** To learn whether different levels of p27 protein were determined at a transcriptional level in our lung cancer specimens, we performed Northern blot analysis. Fig. 2B shows Northern blot analysis of a representative lung cancer panel including
five specimens with high expression levels of p27 protein (Lanes 1–5) and eight displaying low to undetectable levels (Lanes 6–13) of this protein. Similar amounts of p27 transcript were found to be present in all of the examined specimens (Fig. 2B).

Because we did not find regulation of the p27 protein at the transcriptional level and because alteration of p27 never has been detected at the nucleic acid level, we decided to perform degradation assays to learn if the p27 degradation pathway was enhanced in tumors with low to undetectable p27 expression. One g of each frozen human tissue sample (12 representative samples of the 108 specimens analyzed by Western blot) was sectioned and quickly homogenized at 15,000 rpm in 1 ml of ice-cold, doubly distilled water. Each sample was frozen and thawed three times. Purified recombinant p27 was incubated either alone or in the presence of extracts from tumor expressing different p27 levels for 1, 3, 6, 9, and 12 h. Tumors with high p27 expression level presented lower degradation activity compared with tumors with low or undetectable p27 expression level that presented high or very high degradation activity (Table 1). Representative kinetic profiles of p27 degradation are shown in Fig. 2C. In panel a, an extract from a tumor expressing low levels of p27 starts to degrade the purified recombinant p27 after the first hour of incubation, and at 12 h, the p27 protein is completely degraded. Instead, in panel b, an extract from a tumor expressing high levels of p27 has a dramatically lower effect on the degradation of purified recombinant p27 protein.
To demonstrate that the proteasome was involved in the degradation assays of the sample used previously, we subjected the sample with low to undetectable p27 expression to ultracentrifugation to eliminate the proteasome particles. Purified recombinant p27 was incubated in the presence of extracts from the proteasome-depleted supernatant and in the same supernatant after readdition of the proteasome-rich pellet. In addition, the purified recombinant p27 was incubated either alone or in the presence of extracts from tumor expressing low p27 levels as controls (Fig. 2D). No degradation was detected in the sample in which the proteasome was left out. p27 degradation was found to be restored in the sample in which the proteasome was added to the supernatant.

Low Levels of p27 Protein in Non-Small Cell Lung Cancer Correlate with Poor Prognosis. We divided our specimens into three groups of tumors based on the coded score described previously to obtain statistically significant results. The first group (non-expressors) contained 12 specimens (11.1%) with up to 5% of cells positive for p27; the second group (low expressors) contained 56 (51.8%) specimens with up to 50% of positive cells. The third group contained 40 specimens (37%) with p27 immunostaining detected in more than 50% of positive cells (high expressors). Analysis of the data using such necessarily arbitrary cut-offs was highly statistically significant and, therefore, functionally operative.

When we analyzed our results by statistical methods, age, gender, grading, and Tumor-Node-Metastasis status did not correlate with p27 expression levels or with survival. p27 expression was associated significantly with overall survival (Fig. 3). The median survival time was 20 months in low expressor patients compared to 30 months in high expressor patients and 14 months in non-expressor patients. The 4-year survival rate statistically differed among the three groups ($P = 0.0012$). The low expressor group had an overall survival of 14% compared to 25% for the high expressor group ($P = 0.0282$). All 12 non-expressor patients were dead within 30 months following surgery. When the relationship between p27 expression and survival was evaluated statistically for each stage group, no significant difference between the median and overall survival of high expressor patients compared with low and non-expressor patients was found. This is probably due to the small number of patients included in each group.

Discussion

CKIs act as cell cycle-negative regulators through the inhibition of CDKs. It has been suggested that some of these proteins may be involved in human cancer pathogenesis. For instance, $p16$ and $p15$ genes have been found altered in a large number of human neoplasms (21). $p21$ altered expression has been reported in colorectal, pancreatic, and lung neoplasms (22–24).

### Table 1 Correlation between p27 expression levels and p27 degradation activity

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*Protein levels assessed by immunoblot were scored for the intensity of the signal:
  ++ ++, very high signal; ++, moderate signal; +, low signal; and –, undetectable signal! The relative intensity of the immunoblotting signals was in agreement with the immunohistochemical findings.*

The $p27$ gene has not been found mutated in human tumors (11–16), but recent evidence suggests an involvement of this CKI in neoplastic transformation. It has been reported that $p27$-deficient mice develop tumors of the pituitary gland with 100% of penetrance (25, 26). In addition, $p27$ has been shown to be a target of adenovirus E1A (27), and human papillomavirus E7 oncoproteins can dissociate $p27$ from the cyclin/CDK complexes (28).

We investigated $p27$ protein expression in a series of 108 non-small cell lung cancer specimens to verify whether the lack or the reduced expression of this protein was related to neoplastic transformation. We used a double approach to detect $p27$ protein expression. We performed immunohistochemistry and Western blot analysis on each specimen, and we found a very tight correlation between the results obtained with both methods ($P < 0.001$).

It has been demonstrated that $p27$ abundance is mostly regulated at a post-transcriptional level by ubiquitin-proteasome-mediated proteolysis (9). Our results show that $p27$ degradation was enhanced in the samples expressing low/absent $p27$. An indirect confirmation of these results is the detection of the normal $p27$ transcript in all of our specimens. Similar results have been also reported in colorectal carcinomas (19).

When we statistically evaluated the outcome of the patients and correlated it to $p27$ expression, we found $p27$ to be an independent prognostic factor correlating with the overall survival time of the patients ($P = 0.0012$). Similar results have been obtained for $p27$ in a cohort of 149 patients with carcinoma of the colon or rectum (19).

In the absence of a widely accepted prognostic marker for non-small cell lung cancer, demonstration of the prognostic value of a simple assay, such as the immunohistochemical analysis of the $p27$ status on routinely formalin-fixed, paraffin-embedded specimens, acquires a high value and highlights a putative new target for molecular therapeutic approaches to this kind of neoplasia. However, further studies are required to compare the prognostic value of $p27$ immunodetection to other markers proposed recently, such as K-ras, p53, or erbB2. In addition, confirmation in lung cancer of the involvement of proteasome-mediated proteolysis in $p27$ degradation suggests the use of the degradation assay as an additional prognostic method and stimulates new strategies of therapeutic intervention.

Acknowledgments

We thank Professor W. Hauck for statistical analyses. We extend our appreciation to G. Sgaramella for his many contributions in helping to set up...
the experimental conditions. We also thank P. Centonze and C. Dean for technical assistance.

References


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