Low Levels of p27 in Association With Deregulated p53-pRb Protein Status Enhance Tumor Proliferation and Chromosomal Instability in Non-Small Cell Lung Carcinomas

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Abstract

Background: Down-regulation or overexpression of the cyclin-dependent kinase inhibitor p27 have been observed in a range of malignancies, including lung cancer. To further elucidate the role of the molecule in tumor growth regulation, we evaluated p27 expression in a series of non-small cell lung carcinomas (NSCLCs), and examined its relation with histology, kinetic parameters, ploidy, and overall survival. We extended our investigation into the association of p27 levels with the presence of Ki-ras mutations, as well as with the expression status of p53 and pRb in tumor cells.

Material and Methods: p27, p53, and pRb status were immunohistochemically evaluated in a total of 69 NSCLCs. In situ assays were employed to assess the kinetic parameters (Ki-67 immunohistochemistry for proliferation index, Tdt-mediated dUTP nick end labeling assay for apoptotic index). The ploidy status of the tumors was assessed after staining nuclei with the Feulgen procedure, and the presence of Ki-ras mutations was examined by restriction fragment length polymorphisms. All possible associations were assessed with a series of statistical methods.

Results: Immunoreactivity for p27 was observed in the entire series of specimens, with the mean percentage of positive cells being 33%. Adenocarcinomas (AdCs) exhibited higher p27 levels compared to squamous cell carcinomas (SqCCs) (p < 0.01). An inverse correlation was established between p27 expression and proliferation index (PI) (r = −0.834, p < 0.01) but not with apoptotic index (AI), whereas aneuploid tumors were characterized by lower p27 levels than diploid ones (p < 0.01). No difference in p27 immunostaining was observed with regard to the presence of Ki-ras mutations, whereas aberrant p53 and/or pRb expression patterns were associated with p27 underexpression (p < 0.01 for p53 status, p < 0.05 regarding pRb levels, and p < 0.01 for a combined deregulation of both proteins). Two or more alterations in the p27/p53/pRb protein network (i.e., p27 levels lower than the estimated mean value, overexpressed p53, and/or aberrant pRb) were associated with increased PI and aneuploidy (p < 0.001 and p < 0.01, respectively). A powerful trend was found between p27 expression and overall survival (p = 0.066).

Conclusions: Our findings confirm the heterogeneity between AdCs and SqCCs, and are suggestive of an increased proliferative activity in NSCLCs underexpressing p27. Furthermore, our analysis supports the concept of p27 forming a functionally compact network with p53 and pRb, which is actively involved in the regulation of cellular proliferation and chromosomal stability.

Introduction

Complexes composed of cyclins and cyclin-dependent kinases (CDKs) have been shown to control cell-cycle progression through an ordered series of events. In this regulatory process, cyclin-dependent kinase inhibitors (CDKIs), which inactivate the aforementioned complexes, are classified into two families according to their structural and functional characteristics. The INK4 family of CDKIs contains p16, p15, p18, and p19/20, which form binary complexes with CDK4 and/or CDK6, as well as D-type cyclins. The second family, designated the CIP/KIP group, includes p21, p27, and p57, which are able to inhibit all types of CDKs. These proteins, also known as “universal” CDKIs, appear to control cel-
lular proliferation by blocking cell-cycle progression in response to various anti-mitogenic signals (1).

Overexpression of p27 has been demonstrated in quiescent cells, whereas proliferating cells are characterized by lower levels of the protein (2). The gene encoding p27 has been mapped at 12p12-12p13.1 (3) and its product inhibits the function of cyclin E–CDK2 complexes, and represses the transcriptional activation of the cyclin A gene, thus resulting in G1 arrest (4,5). These features of p27 imply that it may act as an oncosuppressor protein. Loss of p27 expression seems not to result from genetic alterations (3,6). Protein levels are mainly regulated post-translationally via ubiquitin-mediated proteolysis (2,4). Recently, the participation of activated Ras in this process has been suggested (7).

Over the last 5 years, down-regulation of p27 expression has been demonstrated in a number of neoplasms, such as endocrine tumors, breast, colorectal, and ovarian carcinomas, and non-Hodgkin’s lymphomas, and is proposed as a poor prognostic factor (Reviewed in Lloyd et al. [1]). In lung cancer, low levels of p27 have been reported in non-small cell lung carcinomas (NSCLCs) with various positivity percentages and immunostaining patterns (8–13), whereas in small cell lung carcinomas (SCLCs), p27 has been found up-regulated (9).

The latter observation, along with a current study demonstrating high levels of p27 expression in a subset of aggressive B-cell lymphomas, contrasts the postulated role of p27 as a tumor suppressor protein (14). Furthermore, Shoji et al. (11) have reported an association of p27 with Ki-67 in lung adenocarcinomas (AdCs), suggesting that p27 may, in some cases, act as a positive regulator of proliferation.

In view of these controversial findings, we comprehensively investigated the relation of p27 expression with the proliferative, apoptotic, and ploidy status of the tumors, as well as the presence of ras mutations, in a series of 69 NSCLCs. We also examined whether alterations in the cell-cycle regulators p53 and pRb correlate with p27 protein levels, and evaluated the impact of combined aberrant patterns on tumor kinetics and chromosomal stability. To the best of our knowledge, a similar study has not been conducted thus far.

Materials and Methods

Tissue Samples

A total of 69 NSCLCs were obtained from surgically treated patients in less than 15 min. Snap frozen samples in liquid nitrogen stored at −70°C, and formalin-fixed and paraffin-embedded (FFPE) material was available. The patients had not undergone any chemo- or radiotherapy prior to surgical resection, thus avoiding up- or down-regulation of cell cycle proteins due to DNA damage (15). The material comprised 33 adenocarcinomas (AdCs) 31 squamous cell carcinomas (SqCCs), and 5 undifferentiated large cell carcinomas (ULCs). Consecutive 5 μm paraffin sections were obtained from each tumor specimen for immunohistochemical evaluation. Representative hematoxylin-eosin stained sections from each tumor lesion were examined by histopathologic examination to confirm that all tumor samples contained 60% or more tumor cells. Tumors were classified according to the World Health Organization criteria and TNM system (16). The clinicopathologic features of the patients are presented in Table 1.

Immunohistochemistry

Antibodies. For immunohistochemical analysis the following antibodies (Abs) were used: SX53G8 (class: IgG1 mouse monoclonal antibody [Mab], immunogen: GST-p27Kip1 fusion protein) (Dako, Athens, Greece), MIB-1 (class: IgG1, mouse monoclonal, immunogen: Ki-67 nuclear antigen) (Oncogene Science, Biodynamics, Athens, Greece), LM95.1 (class: IgG mouse Mab, immunogen: pRb) (Calbiochem, Athens, Greece), DO-7 (class: IgG2b mouse Mab, immunogen: residues 1-45 of p53) (Dako).

Method. Immunohistochemistry was performed according to the indirect biotin–streptavidin–peroxidase method, as previously described (17). All antibodies were used in a 1:100 dilution.

Controls. To discriminate false positive from positive background cells, we performed an additional control assay in which each immunostaining assay step was sequentially eliminated.

Evaluation.

p27 For scoring p27 expression, tumor cells stained at the nucleus were considered positive and the percentage of p27 positive cells in the whole tumor region was assessed.

Ki-67 Tumor cells were evaluated as positive when nuclear staining, without cytoplasmic background, was observed. Proliferative index (PI) was estimated as the percentage of Ki-67 positive cells in 7–10 high power fields (HPF) (total counted cells: about 1000).

tpRb Cytoplasmic reactivity was disregarded and only nuclear staining above any cytoplasmic background was considered as evidence of protein expression. The samples were divided into two categories: normal (No), in which more than 90% of the tumor nuclei were stained, and abnormal (Ab), when there was absence of nuclear staining in a portion of (heterogeneous) or in the entire (homogeneous) tumor section. In addition, admixed non-neoplastic cells showed nuclear reactivity. A mosaic
Table 1. Summary of p27, Ki-ras, pRb and p53 status, kinetic parameters, ploidy and clinicopathologic features

| Patient | p27 Status (%) | Ki-ras Mutations | pRb Status | p53 Status | PI (%) | AI (%) | GI (%) | Ploidy | Histology | LN | Stage | Survival Status (Months after surgery) |
|---------|----------------|------------------|------------|------------|--------|--------|--------|--------|-----------|----|-------|---------------------------------------|
| 1       | 25             | Ab               | p           | 31.1       | 4.8    | 6.5    | A      | AdC     | yes       | III| 3     | 3                                     |
| 2       | N/A            | no               | N/A        | N/A        | NI     | NI     | NI     | SqC     | yes       | III| 21    |                                       |
| 3       | 50             | no               | No         | 28.3       | 1.6    | 17.7   | A      | SqC     | no        | I  | 37*   |                                       |
| 4       | 8              | no               | Ab         | p          | NI     | NI     | NI     | A       | ULC     | no       | I  | 0     |                                       |
| 5       | 30             | N/A              | No         | p          | 32.8   | 6.3    | 5.2    | A       | AdC     | yes      | III| 12    |                                       |
| 6       | 20             | Ab               | p          | 45.6       | 0.6    | 76.0   | A      | SqC     | yes      | II | 35*   |                                       |
| 7       | 50             | N/A              | No         | p          | 27.2   | 1.2    | 22.7   | A       | SqC     | yes      | II | 35*   |                                       |
| 8       | 38             | N/A              | No         | p          | 31.0   | 1.4    | 22.1   | D       | AdC     | no       | I  | 34*   |                                       |
| 9       | 43             | no               | Ab         | p          | 30.3   | 2.0    | 15.2   | D       | AdC     | no       | I  | 35*   |                                       |
| 10      | 20             | no               | No         | p          | 40.6   | 1.8    | 22.6   | A       | ULC     | yes      | II | 9     |                                       |
| 11      | 28             | no               | No         | p          | 36.0   | 0.7    | 51.4   | A       | SqC     | no       | I  | 34*   |                                       |
| 12      | 32             | N/A              | Ab         | p          | 29.5   | 0.9    | 32.8   | A       | SqC     | no       | I  | 35*   |                                       |
| 13      | 38             | no               | No         | p          | 32.8   | NI     | NI     | D       | SqC     | yes      | III| 8     |                                       |
| 14      | 27             | no               | Ab         | p          | 38.5   | 1.3    | 29.6   | A       | SqC     | yes      | III| 8     |                                       |
| 15      | 19             | 2                | Ab         | p          | 40.6   | 1.1    | 36.9   | A       | SqC     | no       | I  | 34*   |                                       |
| 16      | 68             | no               | No         | p          | 4.6    | 0.8    | 5.8    | D       | AdC     | no       | I  | 7     |                                       |
| 17      | 28             | no               | No         | p          | 27.3   | 1.2    | 22.8   | Ni      | SqC     | no       | II | 34*   |                                       |
| 18      | 19             | no               | No         | p          | 43.6   | 4.8    | 9.1    | D       | SqC     | yes      | II | 11    |                                       |
| 19      | 28             | no               | No         | n          | NI     | 10.6   | NI     | D       | AdC     | no       | I  | 34*   |                                       |
| 20      | 46             | no               | No         | n          | 24.6   | 3.5    | 7.0    | D       | SqC     | no       | I  | 34*   |                                       |
| 21      | 30             | no               | No         | n          | 34.5   | NI     | NI     | D       | SqC     | yes      | III| 1     |                                       |
| 22      | 44             | 1                | No         | n          | 46.6   | 1.5    | 31.1   | D       | AdC     | no       | I  | 34*   |                                       |
| 23      | 50             | no               | Ab         | n          | 26.7   | 2.6    | 10.3   | D       | AdC     | yes      | II | 18    |                                       |
| 24      | 58             | 2                | No         | p          | 26.7   | 0.8    | 33.4   | NI      | SqC     | no       | I  | N/A   |                                       |
| 25      | 42             | no               | Ab         | n          | 21.3   | 1.4    | 15.2   | D       | AdC     | no       | I  | 33*   |                                       |
| 26      | 36             | no               | No         | p          | 29.2   | 0.1    | 292.0  | A       | AdC     | yes      | II | 17    |                                       |
| 27      | 68             | no               | Ab         | n          | 13.3   | 3.5    | 3.8    | NI      | AdC     | yes      | II | 14    |                                       |
| 28      | 25             | no               | Ab         | p          | 40.9   | 1.5    | 27.3   | A       | SqC     | no       | I  | 33*   |                                       |
| 29      | 21             | no               | Ab         | p          | 43.4   | 2.7    | 16.1   | A       | ULC     | yes      | III| 0     |                                       |
| 30      | 38             | 1                | No         | n          | 28.8   | 5.2    | 5.5    | D       | ULC     | no       | I  | 32*   |                                       |
| 31      | 53             | no               | No         | n          | 21.2   | 3.0    | 7.1    | D       | AdC     | no       | I  | 10    |                                       |
| 32      | 47             | N/A              | No         | n          | 26.4   | 0.7    | 37.7   | A       | AdC     | yes      | III| 30*   |                                       |
| 33      | 20             | N/A              | Ab         | n          | 40.7   | 0.8    | 50.9   | A       | SqC     | no       | I  | 14    |                                       |
| 34      | 38             | N/A              | Ab         | n          | 34.7   | 0.5    | 69.4   | D       | AdC     | no       | I  | 32*   |                                       |
| 35      | 31             | N/A              | Ab         | p          | 29.2   | 0.8    | 36.5   | A       | AdC     | yes      | II | 17    |                                       |
| 36      | 29             | N/A              | No         | n          | 28.8   | 99.0   | NI     | A       | AdC     | yes      | III| 10    |                                       |
| 37      | 60             | no               | No         | n          | 28.9   | 3.2    | 9.0    | A       | AdC     | yes      | II | 12    |                                       |
| 38      | 32             | N/A              | No         | p          | 39.3   | 3.0    | 13.1   | A       | SqC     | yes      | III| 30*   |                                       |
| 39      | 8              | no               | No         | n          | 54.0   | 0.9    | 60.0   | D       | SqC     | yes      | III| 9     |                                       |
| 40      | 33             | N/A              | No         | n          | 31.0   | NI     | NI     | A       | ULC     | no       | I  | 18    |                                       |
| 41      | 31             | N/A              | No         | n          | 38.0   | 0.5    | 76.0   | A       | SqC     | no       | I  | 30*   |                                       |

(Continued)
pattern of staining with absence of immunoreactivity in a proportion of tumor cells was not interpreted as abnormal (18,19).

**p53** Tumors were considered p53 positive when more than 20% of the tumor cells showed nuclear staining and the remaining tumors were scored as negative (18,19). Slide examination was performed by three independent observers (VG, PZ, CK). Interobserver variability was minimal ($p < 0.01$ by $\chi^2$ test).

**Ki-ras Mutation Analysis**

**RNA Extraction and cDNA Preparation.** Total RNA was extracted from tissues with TriZol reagent (Life Technologies, Anti-Sel, Athens, Greece) following manufacturer's instructions. Quantity and quality of RNAs was assessed by spectrophotometry and agarose gel electrophoresis (20). Two micrograms of total RNA were reverse transcribed with oligo-dT (NEB, BioLine, Athens, Greece) and 200U MMLV reverse transcriptase (Life Technologies, Anti-Sel) according to supplier's instructions.

| Patient | p27 Status (%) | Ki-ras Mutations | pRB Status | p53 Status | PI (%) | AI (%) | GI (%) | Ploidy | Histology | LN | Stage | Survival Status (Months after surgery) |
|---------|----------------|------------------|------------|------------|--------|--------|--------|--------|-----------|----|-------|---------------------------------------|
| 42      | 15 no          |                  | No p       | 43.8       | 0.6    | 73.0   | A      | SqC yes | III       |     | 30*   |                                       |
| 43      | 42 no          |                  | No n       | 21.4       | 3.5    | 6.1    | A      | AdC yes | II        |     | 30*   |                                       |
| 44      | 43 1 no        |                  | No n       | 22.3       | 0.7    | 31.9   | A      | AdC yes | III       |     | 14    |                                       |
| 45      | 21 1 no        |                  | No n       | 32.9       | 8.9    | 3.7    | D      | AdC yes | II        |     | 27    |                                       |
| 46      | 22 no          |                  | No n       | 18.1       | 1.4    | 12.9   | D      | AdC yes | II        |     | 27    |                                       |
| 47      | 8 no           |                  | Ab p       | 54.4       | 0.5    | 108.8  | A      | SqC no  | I         |     | 25    |                                       |
| 48      | 19 1 no        |                  | Ab p       | 44.0       | 0.5    | 88.0   | D      | SqC no  | III       |     | 14    |                                       |
| 49      | 25 1 no        |                  | Ab n       | 36.9       | 1.4    | 29.6   | D      | SqC no  | I         |     | 29*   |                                       |
| 50      | 54 2 no        |                  | No n       | 18.1       | 1.4    | 12.9   | D      | AdC yes | II        |     | 27    |                                       |
| 51      | 52 no          |                  | No n       | 20.0       | 2.9    | 6.9    | D      | AdC yes | II        |     | 15    |                                       |
| 52      | 30 1 N/A       |                  | No p       | 39.0       | 0.6    | 65.0   | A      | SqC no  | III       |     | 29*   |                                       |
| 53      | 25 1 no        |                  | Ab p       | 51.2       | 2.6    | 19.7   | A      | SqC yes | III       |     | 15    |                                       |
| 54      | 31 1 no        |                  | No n       | 32.2       | 3.1    | 10.4   | NI     | SqC no  | I         |     | 28*   |                                       |
| 55      | 25 no          |                  | Ab p       | 43.3       | 0.8    | 54.1   | A      | AdC yes | II        |     | 10    |                                       |
| 56      | 30 1 Ab        |                  | Ab p       | 47.3       | 0.4    | 118.3  | A      | SqC yes | II        |     | 11    |                                       |
| 57      | 32 no          |                  | Ab p       | 40.0       | 1.8    | 22.2   | A      | SqC yes | III       |     | 8     |                                       |
| 58      | 25 1 no        |                  | Ab p       | 46.0       | 0.6    | 76.7   | A      | AdC yes | II        |     | 26*   |                                       |
| 59      | 33 1 no        |                  | No p       | 30.4       | 1.4    | 29.6   | A      | SqC no  | I         |     | 25    |                                       |
| 60      | 20 1 Ab        |                  | Ab p       | 38.6       | 1.0    | 38.6   | A      | AdC yes | III       |     | 23    |                                       |
| 61      | 40 2 No        |                  | Ab p       | 24.5       | 1.1    | 22.3   | D      | AdC yes | III       |     | 26*   |                                       |
| 62      | 25 1 N/A       |                  | No n       | 41.4       | 1.4    | 29.6   | A      | SqC no  | I         |     | 13    |                                       |
| 63      | N/A no         |                  | Ab p       | 50.6       | 1.0    | 50.6   | A      | AdC no  | III       |     | 25*   |                                       |
| 64      | 33 1 no        |                  | Ab p       | 39.3       | 1.6    | 24.6   | D      | AdC no  | I         |     | 25*   |                                       |
| 65      | 10 no          |                  | Ab p       | 70.4       | 0.7    | 100.6  | A      | SqC yes | II        |     | 22    |                                       |
| 66      | 30 2 No        |                  | No n       | 33.3       | 2.3    | 14.5   | A      | SqC yes | II        |     | 3     |                                       |
| 67      | 51 no          |                  | No p       | 20.2       | 0.6    | 33.7   | D      | AdC no  | I         |     | 24*   |                                       |
| 68      | 8 1 no         |                  | No n       | 59.1       | 2.1    | 28.1   | A      | SqC yes | II        |     | 21    |                                       |
| 69      | 30 no          |                  | No p       | 59.1       | 0.9    | 59.1   | A      | AdC yes | II        |     | 23    |                                       |

**Table 1.** (Continued)

**Abbreviations:** N/A, data not available; no mutations; 1, mutation at codon 12; 2, mutation at codon 13; NI, non-informative; Ab, aberrant expression; No, normal expression; p, positive expression; n, negative expression; PI, proliferation index; AI, apoptotic index; GI, growth index; A, aneuploid; D, diploid; SqC, squamous cell carcinoma; AdC, adenocarcinoma; ULC, undifferentiated large cell carcinoma; LN, lymph node invasion; *, patient still alive.
Ki-ras Mutation Detection. Codons 12 and 13 of Ki-ras were screened for mutations in our database. Briefly, polymerase chain reaction (PCR) was performed on cDNA by introduction of artificial Restriction Fragment Length Polymorphism (RFLP) sites for detection of codon 12 mutations, whereas the most common mutation at codon 13 (Gly to Asp) was detected by employing a naturally occurring RFLP (21). The cell lines SW480 (human colon carcinoma) and MDA-MB231 (human breast cancer) carry Ki-ras mutations at codons 12 and 13, respectively, and were used as positive controls for Ki-ras mutation analysis (see Kotsinas et al. [21] and references therein).

TdT-Mediated dUTP Nick End Labeling Assay
Method Double-strand DNA breaks were detected by TdT-mediated dUTP nick end labeling (TUNEL) assay according to the procedure proposed by Gavrieli et al. (22).

Controls As negative control, tissue sections incubated with TdT buffer without enzyme were used. As positive control, tissue sections incubated with DNase I for 45 min prior to treatment with TdT were used.

Evaluation Cells were considered to undergo apoptosis where nuclear staining, without cytoplasmic background, was observed. Apoptotic index (AI) was estimated as the percentage of apoptotic cells in 7–10 HPFs (total counted cells: about 1000). Slide examination was performed by three independent observers (VG, PZ, CK). Interobserver variability was minimal (p < 0.01 by χ2 test).

DNA Ploidy Analysis
Method. The samples were stained using the Thionin-Feulgen procedure (21).

Evaluation. The measuring procedure was performed using the Optipath TV-based image analysis system (Meyer Instruments of Houston, Houston, Texas, USA) equipped with a microscope and a video CCD camera. As internal reference control, lymphocytes or granulocytes were used. In each analysis, approximately 100 control and 350 tumor cells were measured. To distinguish nondiploid from diploid cells, an upper limit of 2.5 c was set for diploid values. Because the fraction above 2.5 c might include proliferating diploid cells, we also calculated the fraction of tumor cells with DNA values above the 5 c level, which exceeds those of proliferating diploid cells. Cases with more than 5% of cells with DNA content above 5 c were considered as aneuploid, as previously described (23).

Statistical Analysis
We used the logarithms of the apoptotic and growth indexes (GI: the ratio of PI to AI) to normalize the variables. The possible relations between p27 and PI, AI, or GI were assessed by the parametric Pearson’s test. Differences in p27 labeling indices (LIs) between tumors with different histologic, ploidy, lymph node, p53/pRb, and ras mutational status were analyzed by the t-test. Association between p27 levels with tumor stage and the p53/pRb status was estimated by analysis of variance (ANOVA). For multiple comparisons among alterations of the p53/pRb status and p27 levels, as well as among aberrations in the entire protein network and the kinetic parameters of the tumors, the Scheffe test was performed. Finally, the association between ploidy and alterations in the above network was estimated by the χ2 test, and the Kaplan-Meier methodology was employed for assessing the survival curves of the parameter examined in the present study. All analyses were performed with the SPSS statistical package on a personal computer. Differences were considered significant when p < 0.05.

RESULTS
Correlation of p27 Expression With Clinicopathologic Features
Immunoreactivity for p27 was found both in normal and cancerous cells. The signal was mostly nuclear and cytoplasmic staining observed in some cases was not evaluated (Fig. 1A). Normal bronchial epithelial cells, as well as infiltrating lymphocytes, exhibited strong immunostaining and served as internal positive controls. In some well-differentiated AdCs, immunoreactivity was lower at the center of tumor nodules, whereas in SqCCs, p27 expression was often higher toward the central tumor regions (Fig. 1B). The mean percentage of cells expressing p27 was 32.7 ± 13.96% in the examined tumors (range 8–68%). The difference in p27 levels between AdCs and SqCCs was highly significant (mean value 38.94 ± 13.21% versus 27.47 ± 12.40%, respectively; p < 0.01). p27 immunostaining was not associated with lymph node metastases or the stage of the tumors (Table 2).

Correlation Between p27 Expression, Kinetic Parameters, and Ploidy Status of the Tumors
The proliferative activity of tumor cells was assessed by Ki-67 immunohistochemistry. The MIB-1 antibody recognizes a nuclear non-histone protein, which is expressed throughout the cell cycle, but not in G0, thus providing a representative marker of proliferation (24). The mean percentage of positive cells for Ki-67 in the cancerous areas was 34.8 ± 11.29%. An inverse correlation between p27 immunoreactivity and PI was
Fig. 1. Representative results of p27 immunohistochemical staining. Streptavidin–biotin–peroxidase technique with the SX53G8 anti-p27 antibody (DAB as chromogen, see “Materials and Methods) and hematoxylin counterstain. (A) Adenocarcinoma (case 59) with intermediate p27 staining (33%) ×400. Large, thick arrow indicates a stained tumor nucleus; small, thick arrow shows a stained lymphocyte nucleus. Thin arrow points out a stained fibroblast nucleus. (B) Squamous cell carcinoma (case 24) with high p27 staining (58%) ×200.

Table 2. Relationship between p27 expression, clinicopathologic parameters, pRb status, p53 status and Ki-ras mutations

| Parameter                | Number | p27 Mean Value | p   |
|--------------------------|--------|----------------|-----|
| Histology                |        |                |     |
| AdCs                     | 32     | 38.94 ± 13.21  | 0.001|
| SqCCs                    | 30     | 27.47 ± 12.40  |     |
| Ploidy                   |        |                |     |
| Aneuploid                | 39     | 28.33 ± 11.59  | 0.007|
| Diploid                  | 24     | 37.50 ± 14.07  |     |
| LN                       |        |                |     |
| Yes                      | 36     | 31.75 ± 14.33  | 0.558|
| No                       | 31     | 33.77 ± 13.67  |     |
| Tumor stage              |        |                |     |
| I                        | 28     | 34.64 ± 14.04  | 0.309|
| II                       | 21     | 33.76 ± 16.47  |     |
| III                      | 18     | 28.39 ± 9.86   |     |
| pRb status               |        |                |     |
| Aberrant                 | 26     | 27.73 ± 13.05  | 0.02 |
| Normal                   | 41     | 35.83 ± 13.75  | 0.009|
| Positive                 | 39     | 28.97 ± 13.03  |     |
| p53 status               |        |                |     |
| Negative                 | 28     | 37.86 ± 13.78  | 0.582|
| Yes                      | 14     | 34.71 ± 12.14  |     |
| Ki-ras mutations         |        |                |     |
| No                       | 38     | 32.05 ± 16.36  |     |
| Normal                   | 22     | 37.14 ± 13.01  | 0.002|
| 1 alteration             | 25     | 35.80 ± 15.30  |     |
| 2 alterations            | 20     | 23.90 ± 8.79   |     |

Abbreviations: AdCs, adenocarcinomas; SqCCs, squamous cell carcinomas.
Thirty nine of 63 p27-informative tumors (61.9%) were scored as aneuploid, and were characterized by significantly lower p27 levels than diploid ones (28.33 ± 11.59% versus 37.50 ± 14.07%, respectively; p < 0.01; see Table 2).

Association of p27 Levels With Ki-ras Mutations, p53, and pRb Expression Status

Ki-ras mutations were detected in 14 of 52 p27-informative tumors examined (26.9%) (Table 1, Fig. 3). No relation was observed between p27 immunoreactivity and the presence of such mutations.

Aberrant pRb expression was detected in 26 of 67 p27-informative tumors (38.8%), with p27 levels being significantly lower in specimens with aberrant than normal pRb status (27.73 ± 13.05% versus 35.83 ± 13.75%, respectively; p = 0.05; see Table 2). Moreover, overexpression of p53 (statistically associated with p53 mutation) (18,19) was found in 39 out of 67 p27-informative tumors (58.2%). Specimens overexpressing p53 correlated with reduced p27 levels compared to negative tumors for p53 expression (28.97 ± 13.03% for p53-positive versus 37.86 ± 13.78% for p53-negative specimens, p < 0.01; see Table 2). Concerning the combined p53/pRb status, p27-informative tumors were classified into three groups. The first group, possessing no aberrations, contained 22 specimens (32.8%). The second group, showing one alteration in either pRb or p53 status, included 25 cases (37.3%). The third group, exhibiting alterations of both pRb and p53, consisted of 20 (29.9%) specimens. p27 levels were significantly lower in specimens of group 3 compared to the other two (mean difference −13.24 ± 3.98%, p < 0.01 for the first and −11.90 ± 3.87%, p < 0.05 for the second group by ANOVA; see Table 2).

The mean GI value was 38.2 ± 44.09%, with a significant inverse correlation being established between GI and p27 immunostaining for the entire group of tumors (r = −0.470, p < 0.01). Interestingly, when histologic types were separately analyzed, the correlation remained statistically significant for SqCCs (r = −0.454, p < 0.05), but not for AdCs. The correlation coefficients between p27 expression and the kinetic parameters of the tumors are presented in Table 3.

![Fig. 2. Adenocarcinoma (case 9) with moderate apoptotic index. Tdt-mediated dUTP nick end labeling assay (TUNEL) (DAB as chromogen, see “Materials and Methods”) and hematoxylin counterstain ×200. Arrow indicates an apoptotic nucleus.](image)

**Table 3.** Correlation coefficients between p27 expression frequencies and kinetic parameters of tumors

| Tumor kinetic parameters | AdCs     | SqCs     | All specimens |
|--------------------------|----------|----------|---------------|
| Proliferation index      | −0.749** | −0.823** | −0.834**      |
| Apoptosis index          | −0.020   | 0.201    | 0.119         |
| Growth index             | −0.362   | −0.454*  | −0.470*       |

*p < 0.05.  
**p < 0.01.

![Fig 3. Representative results of Ki-ras mutation (codon 12) analysis. M, 100 bp ladder; C, control PCR fragment before digestion (357 bps). Lanes 1, 2, 3, 5, and 6: BstNI restriction fragment length polymorphism (RFLP) results from tumor samples (cases 19, 20, 21, 23, and 24, respectively) without codon 12 mutations (305 bps). Lane 4: BstNI PCR RFLP results of case 22 harboring a codon 12 Ki-ras mutation (335 bps).](image)
Relations of p27/p53/pRb Expression Patterns With the Kinetic Parameters and Ploidy Status of the Tumors

With regard to the number of alterations in the p27/p53/pRb protein network (i.e., p27 levels lower than the estimated mean value, mutant p53, and aberrant pRb status), tumors were divided into four groups, harboring no or one to three aberrations. Specimens with two and three alterations were characterized by a significantly increased PI, compared to tumors with one or no alterations (Table 4). No differences were observed in the AI values among the four groups examined. Moreover, GI was significantly higher in specimens with three alterations compared to specimens with no alterations (mean difference $-0.52 \pm 0.15$, $p < 0.05$ by ANOVA; Table 5). Ploidy was also associated with deregulation of the above network ($p < 0.01$ according to $\chi^2$) (Table 5).

Survival Analysis

During the course of this study (maximal follow up, 37 months; median follow up, 24.5 months), 35 failures and 29 censored cases were recorded. In this analysis, all patients died of disease; two patients who died during surgery (cases 4 and 29) were excluded. Also, we had no survival data for case 24 and no p27 data for cases 2 and 63; therefore, the analysis was performed with 64 patients. Furthermore, we had no access to data regarding postoperative treatment that could help us in this analysis. The use of the p27 median value as a cut off point revealed a powerful trend ($p = 0.066$). In the first group, 32 patients were included with p27 expression up to 30%; 11 of them were censored and 21 died (mean and median survival time, 21 months). In the second group, 32 patients were included with p27 expression higher than 30%; 18 were censored and 14 died (mean and median survival time, 27 and 25 months, respectively).

Table 4. PI differences between alterations in the p27-p53-pRb network

| Alterations | 0  | 1  | 2  | 3  |
|-------------|----|----|----|----|
|Alterations  | 2.95 | 12.66* | 16.71** |

* $p < 0.05$.  ** $p < 0.001$.

Table 5. Association between alterations in the p27-p53-pRb network, tumor kinetics, and ploidy

| Alter. | PI N Mean value $p$ | AI N Mean value $p$ | GI N Mean value $p$ | Ploidy Aneup. Dip. $p$ |
|--------|---------------------|---------------------|---------------------|------------------------|
| 0      | 12 26.47 ± 7.55     | 11 0.31 ± 0.29      | 11 1.09 ± 0.32      | 6 6                    |
| 1      | 21 29.41 ± 9.99     | 18 0.13 ± 0.47      | 17 1.34 ± 0.49      | 7 13                   |
| 2      | 13 39.13 ± 7.73     | 13 0.18 ± 0.33      | 12 1.38 ± 0.35      | 9 4                    |
| 3      | 17 43.18 ± 9.86     | 17 0.02 ± 0.30      | 17 1.61 ± 0.34      | 17 1                   |

Abbreviations: N; number of samples; PI, proliferation index; AI, apoptotic index; GI, growth index; Alter., alterations; Aneup., aneuploidy; Dip., diploidy.

Discussion

In an attempt to evaluate the impact of p27 expression on tumor growth, we investigated the relation of p27 levels with the kinetic parameters and ploidy status of a series of 69 NSCLCs. To further elucidate the tumorigenic mechanisms at the cellular level, we also examined whether immunoreactivity for p27 correlated with the presence of ras mutations or the expression status of p53 and pRb.

Detectable p27 immunostaining was observed in all specimens, with the mean percentage of positive cells being 32.7%. Furthermore, 59.7% and 40.3% of the carcinomas were characterized by lower and higher values, respectively. Similar percentages have been reported by Yatabe et al. (9). In the majority (83.6%) of tumors, fewer than 50% of cells were found to express p27, in accordance with the study by Catzavelos et al. (12). Contrasting our
results, Esposito et al. (8) reported a fraction (11%) of NSCLCs exhibiting no p27 expression at all, and the mean percentage of p27-positive cells estimated by Kawana et al. (13) was considerably lower than ours, possibly due to tumor sample variability and the antibody used. We mainly obtained nuclear staining; cytoplasmic signals were also observed in some cases and may be associated with the loss of tumor suppressor genes like tuberin (25) or with binding to transcriptional activators, such as Jab1 (26).

Underexpression of p27 represents a frequent finding in human malignancies (1). Furthermore, targeted inactivation of the protein results in hyperplasias of multiple organs and tumorigenesis in vivo (27). Down-regulation of p27 rarely occurs due to point mutations or homozygous deletions (3,6,28), whereas DNA hypermethylation has been shown to predominantly control p27 expression in pituitary cancerous cell lines (29). Translational control may also contribute to the regulation of p27 abundance in contacted inhibited cells and cells undergoing differentiation (30,31), while the principal mechanism involves ubiquitin-mediated proteolysis following p27 phosphorylation (2). Notably, phosphorylation of the protein by cyclin E-CDK2 has been observed in murine fibroblasts, suggesting that this cyclin-CDK complex may promote p27 degradation during cell-cycle progression (4). Moreover, it has been postulated that targeted phosphorylation of p27 by ERKs, which represent well-established Ras effector molecules, may mediate the effects of mitogenic stimuli upon the cell cycle (2). In agreement with this notion, studies in growth factor-stimulated cell lines have demonstrated the involvement of Ras in down-regulation of p27 during late G1, thus allowing entry into the S phase (7). Nevertheless, we found no association between ras mutations and p27 expression, in accordance with the results of Catzavelos et al. (12). Our findings suggest no interference of Ras with p27 activity in NSCLCs.

A significantly lower percentage of cells expressing p27 was observed in SqCCs compared to AdCs. Although decreased p27 levels have been reported in SqCCs (9,10), others have failed to demonstrate a significant difference between the aforementioned histologic types (8). However, the varying expression status of p27 exhibited by different histologic subtypes of thyroid (32), ovarian (33), endocrine (34), and colorectal carcinomas (35), as well as non-Hodgkin’s lymphomas (36), indicate a potential importance of the protein in distinguishing between malignancies with different biological behavior. Furthermore, we found no relation of p27 immunostaining with the presence of lymph node metastases or tumor stage, in accordance with most studies (8,12,13).

A significant correlation was established between GI and immunoreactivity for p27 ($r = -0.470$, $p < 0.01$) for the entire group of specimens. This correlation was sustained for SqCCs ($r = -0.454$, $p < 0.05$), but not for AdCs; the GI determinant of proliferation was significantly correlated with low percentages of p27 only in the SqCC group, whereas the apoptotic determinant, which is discussed later, was similar in both groups. The specific biological profiles of these two NSCLC subtypes may further account for this difference (37). Moreover, according to our results, p27 may represent another discriminating factor between AdCs and SqCCs. Positivity for p27 and Ki-67 was inversely correlated ($r = -0.834$, $p < 0.01$), an observation confirming the results of Kawana et al. (13). Studies conducted in lymphoid, oral, and endocrine tumors (34,38,39) have also yielded similar findings, pointing toward a selective growth advantage of cells underexpressing p27. However, such a correlation has not been established for colorectal and breast carcinomas (35,40,41). Of particular interest is the observation of Shoji et al. (11) that in well-differentiated lung AdCs, immunostaining patterns of p27 and Ki-67 are similar, with expression mostly occurring in the periphery of tumor nodules, in cells possessing an increased proliferative activity. It has been suggested that the association between high cellular p27 levels and increased proliferation may be partially justified by the function of the protein as an assembly factor for the mitotic cyclin A-CDK2 complexes (11) or by the accumulation of inactive p27 in its complexes with cyclin D (14). The latter hypothesis was initially proposed to explain high p27 expression in aggressive B-cell lymphomas (14) and is further reinforced by the observation that cyclin D1-transfected cells may enter S phase without a detectable change in p27 protein levels (4).

In a recent study performed on the same series of lung biopsies, we demonstrated that increased proliferation is associated with p53 mutations and/or aberrant pRb expression (19). We now report that combined deregulation of p27, p53, and pRb exerts a synergistic effect on proliferative activity (Tables 4 and 5), strengthening the suggested concept—delineated in Figure 4—that these cell-cycle regulators form a tight network (1,4,5,15,42,43). In the presence of one abnormality in this protein network, no deviation in the proliferating capacity was observed compared to the fully normal pattern (Table 4). However, upon the occurrence of a second “hit,” proliferation activity increases significantly, reaching even higher levels in cases with the fully abnormal pattern (Table 4, Fig. 5). The putative mechanisms underlying our findings are the following. Down-regulation of p27 (low percentages of p27 positive cells) permits cyclinD-CDK4,6-dependent phosphorylation of pRb (inactive pRb) resulting in S-phase entry (42 and references therein) and additionally releases transcriptional suppression of mitotic cyclin A by disrupting the cyclinE-CDK2/p107-E2F complexes (5). In case p53 is mutated [p53 positive staining (18,19)], its ability to indirectly inhibit cy-
clin-CDK complexes via p21WAF-1, in the stressogenic environment of tumors (e.g., hypoxia, free radicals) is impaired (15,43). One of the consequences of this impairment is accumulation of active cyclinE-CDK2 and consequent down-regulation of p27 (4). Finally, deletion of pRb (aberrant pRb expression) would further confer on proliferation, possibly by deregulating the expression of E2F targeted genes (42 and references therein).

Overexpression of p27\(^{kip1}\) appears to be more cytotoxic to cancer cells compared to that of p21 (44,45), with apoptosis being the putative mechanism underlying such cytotoxicity. However, we found no association between p27 expression and apoptotic index. Tenjo et al. (41) also reported the absence of a correlation between p27 and AI in colorectal AdCs, along with no relation between p27 levels and mt-p53 expression. They have thus suggested that apoptosis in colorectal AdCs is independent of p53 status. In a previous study, we showed that low AI was associated with mt-p53 expression in the same series of lung carcinomas (19). In the present study, we observed that reduction in p27 levels was accompanied with mt-p53. Hence, although the expression status of p27 seems linked to that of p53 in NSCLCs, it appears that p27 is not involved in p53-mediated apoptosis. In contrast to proliferation activity, the apoptotic index was independent of defects in the p27-p53-pRb network (Table 5). Studies in human cancer cell lines have revealed that overexpression of p27\(^{kip1}\) may induce apoptosis through a p53-independent mechanism (45). Enhanced apoptosis has also been observed in p27-null mice, along with increased proliferation and matrix-protein accumulation, and has been related to high CDK2 activity (46). Levkau et al. (47) reported that cleavage of both p27\(^{kip1}\) and p21\(^{cip1/waf1}\) by CPP32 or a CPP32-like caspase leads to their dissociation from nuclear cyclin-CDK2 complexes, with subsequent induction of CDK2 activity in apoptotic human endothelial cells. However, such an event could be tissue specific, and further studies are needed to elucidate the role of p27 in apoptosis.

Expression of p27 was less prominent in aneuploid than diploid tumors. This observation probably stems from the impact of p27 status on the proliferative activity of cancer cells. Because efficient DNA repair occurs during G1, forced entry into the S phase is expected to cause genetic instability. Shortening of the G1 phase may result in additional DNA damage, possibly due to inadequate formation of replication forks, insufficient levels of nucleotides, or diminished activity of ribonucleotide reductase (48 and references therein). The fact that aneuploidy was associated with the full abnormal pattern of the p27-p53-pRb network most likely reflects the effect of such alterations on cellular proliferation.

Finally, a strong trend was observed between the overall survival of the examined group of patients and p27 expression. This finding is in agreement with those of other groups which suggest that p27 may represent an independent prognostic factor for patients’ outcome in lung cancer (8–10,12). Moreover, although Catzavelos et al. (12) found no statistical significance between p27 protein levels and overall survival, they considered p27 expression...
a prognostic marker by evaluating recurrence-free survival data.

In summary, we have shown that p27 expression correlates with histologic type, proliferation activity, and ploidy status, but not with apoptosis, in a series of NSCLCs. A strong trend was found between overall survival and p27 immunoreactivity. Low levels of p27 were associated with expression of mt-p53 and down-regulation of pRb, yet not with the presence of Ki-ras mutations. The most important observation was the cooperative effect of deregulated p27, p53, and pRb expression on tumor proliferation, further supported by the association of aneuploidy with defects in this regulatory network. Investigating the relations of p27 with other molecules implicated in cell-cycle control could enhance our understanding of p27 function in normal and tumor cells.

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