p27kip1 Expression in Non-small Cell Lung Cancer Is not an Independent Prognostic Factor

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Abstract. Background: p27kip1 (p27) plays an important role in cancer cell cycle regulation. Recent evidence however, suggests that p27 may function as an oncogene rather than a tumour suppressor gene. Patients and Methods: Ninety-two patients with previously untreated non-small cell lung cancer (NSCLC) were studied for the association between the immunohistochemical localization of p27 through a semi-quantitative method and time-to-progression (TTP) and overall survival (OS). Results: The relationship between p27 H-Score and both TTP and OS was polynomial. Short TTP in patients with metastasis or whose tumors progressed during the eight-month period after diagnosis was statistically associated with overexpression of PIRH2 (p<0.001). In patients whose tumours progressed later, long TTP was associated with NSCLC of the non-adenocarcinoma type (p=0.027), p27 H-Score (p=0.032) and well-differentiated adenocarcinoma (p=0.047). None of the parameters correlated with duration of OS. Conclusions: This study showed that p27 H-Score may not appear to be an independent prognostic factor in patients with NSCLC.

Lung cancer (LC) ranks among the most commonly occurring malignancies and the leading cause of cancer-related death worldwide (1). Only patients who undergo curative surgery have a significant potential for cure and many tumors diagnosed at an early stage have already spread microscopically. Tumour node metastasis (TNM) staging and histological grading are useful, but not satisfactory classification systems to predict prognosis.

p27kip1 (p27) has attracted much interest as a potential prognostic marker in LC patients because of its important role in cancer cell cycle regulation. p27 prevents or slows down the cyclin–cyclin-dependent kinase (CDK) complex activity in the nucleus (2), and unlike other tumour suppressor genes such as p53 or p16, p27 genomic locus rarely mutates (3). In contrast to the F-box protein SKP2 (which promotes degradation of p27 at early phases of G0-G1 transition), the ring-H2-type ubiquitin ligase PIRH2 regulates p27 at the late G1-S phase, further than the ‘restriction point’ (4). Despite early data supporting the notion that p27 behaves as a tumour suppressor gene, recent evidence suggests that under certain conditions p27 may function as an oncogene (5). Phosphorylation of p27 by oncogenic kinases such as AKT may cause cytoplasmic mislocalisation of p27, associated with inhibition of RhoA activity, augmented cell motility and enhanced metastasis (6). Loss of p27 expression was observed in approximately 30% of LC cases. Expression was higher in adenocarcinoma than in squamous cell carcinoma. While most studies reported an adverse survival effect as a result of the loss of p27 expression (7), a few studies reported inconsistent results either for patients with the non-squamous cell subtypes (8) or all LC patients (9, 10). This discrepancy could be attributed to the inclusion of different groups of patients or non-standardised methodology, although the dual biology of p27 could play a role as well.

In the present study, p27 immunoreactivity was assessed by applying a semi-quantitative method on biopsies of ninety-two LC patients. Patients diagnosed with either early, locally-advanced or metastatic states were grouped together for analyses. In addition, histological grade was evaluated in thirty-nine adenocarcinoma patients, and the expression of PIRH2 and p53 was studied in another twenty-eight patients.

Materials and Methods

Patients. Only newly-diagnosed patients with non-small cell lung cancer (NSCLC) were included in the study. All patients were diagnosed and treated at the SUMC (Soroka University Medical Center) during the years 1998-2008. Only patients for whom high-quality, surgical LC specimens were available were included and all
biopsies were from the original lung tumour. The study dataset consisted of the following groups of variables: (a) demography including age, gender, ethnicity, and place of birth; (b) pathological data including primary tumour size, local invasion, lymph node and distal metastasis according to AJCC classification for lung cancer, 2002 version (11); and (c) clinical data including smoking history, surgery type, treatment mode (chemotherapy or/and radiotherapy) and outcome (tumour progression or survival). Records of patients with pure bronchoalveolar histology were separated from those of adenocarcinoma patients. Tumour size, local invasion and lymph node metastasis were determined by pathological examination. The final disease stage was assessed by a combination of clinical, surgical and pathological findings according to the current TNM staging system for LC. In all cases in which adequate surgery was performed, the pathological staging was the determinant of final disease stage. The study was conducted with the approval of the Ethical Review Board of the SUMC.

Histological examination. Hematoxylin and eosin-stained slides were reviewed for confirmation of histopathological diagnosis and for selection of adequate specimens for analysis. The histological identification of LC was determined as recommended by the World Health Organization (11). Tumours diagnosed as adenocarcinomas were evaluated for grade. When these tumours were composed of cells resembling mature normal lung cells, they were considered as well-differentiated and when these tumours had primitive-appearing cells, they were considered as poorly differentiated.

Immunohistochemistry. Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue sections. Sections 4 μm-thick were deparaffinized with xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase was blocked at room temperature by 3% hydrogen peroxide in methanol for 20 minutes. Heat-induced epitope retrieval was performed in 0.01 M citrate buffer (pH 6.0) and the samples were heated in a microwave oven at 99°C for 20 min. Sections were incubated for 30 min thereafter with 5% blocking horse serum to reduce non-specific binding, and were incubated with the primary antibodies. The following antibodies were used: Anti-p27 (Lab Vision Corporation Neomarkers, Fremont, CA, USA), anti-p53 (Zymed, South San Francisco, CA, USA), and anti-PIRH2 (clone BL588; Bethyl, USA). Diaminobenzidine was used as a chromogen, and hematoxylin was used for counterstaining.

Table I. Demographic, clinical and pathological details of all lung cancer patients.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Frequency or summary statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient’s age at LC diagnosis (years)</td>
<td>Median: 65.2, Range: 40-82</td>
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<tr>
<td>Gender</td>
<td>Male: 69 (75%), Female: 23 (25%)</td>
</tr>
<tr>
<td>Patient’s origin</td>
<td>Africa/Asia: 38 (40%), Israel/Europe/Americas: 37 (38%), Unknown: 17 (22%)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Jewish: 74 (80%), Bedouin/Arab: 7 (8%), Unknown: 11 (11%)</td>
</tr>
<tr>
<td>Smoking</td>
<td>Yes: 75 (82%), No (or unknown): 17 (18%)</td>
</tr>
<tr>
<td>Histological type of LC</td>
<td>Adenocarcinoma: 40 (43%), Squamous cell: 30 (33%), Large cell: 17 (18%), Pure bronchoalveolar: 5 (5%)</td>
</tr>
<tr>
<td>Anatomical stage</td>
<td>I-II: 29 (32%), III: 26 (29%), IV: 35 (39%)</td>
</tr>
<tr>
<td>Adenocarcinoma histological grade</td>
<td>Overall: 39 (100%), Well-differentiated: 24 (61%), Poorly differentiated: 15 (39%)</td>
</tr>
<tr>
<td>Type of surgery</td>
<td>Overall: 33 (100%), Wedge resection: 3 (9%), Lobectomy: 29 (88%), Pneumonectomy: 1 (3%)</td>
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<tr>
<td>Chemotherapy</td>
<td>Overall: 44 (100%), Neoadjuvant: 6 (14%), Concurrent chemo-radiotherapy: 5 (11%), Adjuvant: 4 (9%), Metastatic (or palliative): 29 (66%)</td>
</tr>
<tr>
<td>Type of first-line systemic therapy</td>
<td>Overall: 46 (100%), Platinum-based, doublets: 29 (63%), Single agents (including EGFR-TKIs): 17 (37%)</td>
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<tr>
<td>Recent state of disease</td>
<td>No relapse: 15 (16%), Relapse: 71 (77%), State undetermined: 6 (8%)</td>
</tr>
<tr>
<td>Patient status*</td>
<td>Alive: 22 (24%), Dead: 70 (76%)</td>
</tr>
<tr>
<td>PIRH2 status</td>
<td>Overall: 28 (100%), Positive: 17 (61%), Negative: 11 (39%)</td>
</tr>
</tbody>
</table>

Table I. continued

<table>
<thead>
<tr>
<th>Variables</th>
<th>Frequency or summary statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 status</td>
<td>Overall: 29 (100%), Positive: 17 (59%), Negative: 12 (41%)</td>
</tr>
<tr>
<td>p27 H-Score</td>
<td>Mean±SD: 94.2±83.7, Median: 70</td>
</tr>
<tr>
<td>95% Confidence interval</td>
<td>(0-300)</td>
</tr>
</tbody>
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The sum of percentages may not be equal to 100 because of rounding up.

Table I. continued
Scoring of p27-stained slides. The immunohistochemical localisation of p27 was scored by applying a semi-quantitative method, incorporating both the intensity and the distribution of specific staining as described by Detere et al. (12). No attempt was made for distinctive assessment of p27 in the cytoplasm. A minimum of 500 tumour cells were counted. If differences occurred between spot intensities, the most positive spot was taken into account. The evaluations were recorded as percentages of positively stained target cells in each of the four intensity categories, which were denoted as 0 (no staining), 1+ (weak but detectable above control) and 2+ (distinct), 3+ (strong). For each tissue, a value designating the H-Score was derived by summing the percentages of cells staining at each intensity (Pi) multiplied by the weighted intensity of staining; H-Score=Σ Pi(i+1), where: i=1, 2, 3 and Pi ranges from 0 to 100%.

Statistical analysis. Time to progression (TTP) was calculated as the interval from the surgical diagnosis date to the date of the first recorded progression. For newly diagnosed patients with stage 4 disease, the date of progression was defined as the diagnosis date. In this particular group, TTP was arbitrarily considered to be zero regardless of the following course. This wide definition of TTP enabled p27 H-Score in LC patients of a wide clinical spectrum to be analysed as a single group. Patients alive at the end of the follow-up period or those who died during it of any cause other than LC were censored from the overall survival time (OS) calculation. Survival curves were plotted as the Kaplan-Meier survivor functions.

To analyse the association between the values of the p27 H-Score, TTP and OS time, population-averaged panel-data models were fitted with the data, using generalised estimation equations. Analysis of TTP and OS time also involved fitting univariate and multivariate Cox proportional hazards regression models to the data. To recover missing information, the STATA multiple imputation (MI) method was applied (STATA® software for Windows, release 11.0; StataCorp LP, TX, USA). All statistical tests were two-sided, and statistical significance was defined at a level less than 0.05.

Results

Patient characteristics. Ninety-two patients with a histological diagnosis of NSCLC were included in the study. The demographic, clinico-pathological and immunohistochemical details are presented in Table I.

Immunohistochemistry for p27. The staining intensity of p27 was assessed in both the nucleus and the cytoplasm. p27 stained positively mainly in the nucleus, making nuclear/cytoplasmic ratio assessment difficult. Figure 1 shows three levels of H-Score stain for p27. Negative stain (H-Score=0) is shown in Figure 1A, intermediate level of staining (H-Score=120) is shown in Figure 1B and high level of staining (H-Score=240) is shown in Figure 1C. Figure 2 presents the histogram of the p27 H-Score values for the entire study group. As can be readily seen, the kernel curve, which can be considered as an approximation of the probability distribution of p27 H-Score values, is asymmetrical with a long right-hand tail. The curve reaches its maximum at the median point equal to 70, while the mean value is 91.2 and the standard deviation (SD) is 83.7.

TTP in patients with adenocarcinoma. Changing a predictor variable from continuous to categorical form is a common part of many prognostic variable analyses. For LC patients, the risk of progression may be a function of the continuous variable p27 H-Score. Fractional polynomial regression of TTP was performed on H-Score values to find a possible cut-off point for the p27 H-Score. The resultant regression line in patients with adenocarcinoma is shown in Figure 3. Clearly, this line is not a straight one; in patients with metastasis or whose tumour progressed during the first eight months after diagnosis, the correlation between p27 H-Score and TTP is negative, whereas in patients whose tumours progressed later, the correlation is positive. Because of the non-linear relationship, analysis for TTP was carried out separately for these two categories.

Analysis for TTP in all lung cancer patients. Univariate Cox regressions of TTP were fitted on the following variables: age older than 60 years (yes=1, no=0); male gender (yes=1, no=0); p27 H-score above 100 (yes=1, no=0); PIRH2 status (positive=1, negative=0); adenocarcinoma type (yes=1, no=0); grade of differentiation (well-differentiated=0, poorly differentiated=1) and p53 status (positive=1, negative=0). Among these models, only Cox regression with PIRH2 positive status was found to be statistically significant (p<0.001; Figure 4).
Analysis for TTP in patients with late tumour progression. Applying the condition ‘relapse after the first eight months’, the multivariate Cox regression model of TTP was fitted to the same covariates as in the previous model. Under this condition, the following covariates were statistically significant associated with improved survival (Table II): p27 H-score above 100 ($p=0.034$), non-adenocarcinoma type ($p=0.045$) and well-differentiated adenocarcinoma ($p=0.047$).

Correlation between H-Score and OS duration in patients with adenocarcinoma. In the previous models, the failure event was specified to be tumour progression. In the second part of the analysis, this was replaced by death due to LC. Again, to find a possible cut-off point for the p27 H-Score, fractional polynomial regression of OS time was performed on H-Score values. In patients with p27 H-Score $\leq 60$, the correlation between p27 H-Score and OS duration was negative, but when p27 H-Score was above 60, the correlation was direct.

Analysis for OS duration. The data of only forty patients with adenocarcinoma were fitted to the multivariate Cox regression model of OS time. The following terms were incorporated into the model under the condition ‘p27 H-score $>60$’: age older than 60 years (yes=1, no=0), male gender (yes=1, no=0), PIRH2 status (positive=1, negative=0), adenocarcinoma type (yes=1, no=0), grade of differentiation (well-differentiated=1, poorly-differentiated=0) and p53 status (positive=1, negative=0). It turned out that none of these variables had any significant impact on the duration of OS (data not shown).

Discussion

This study aimed to investigate the association between the expression of p27 in tumours and the prognosis of LC patients. p27 was assessed on using immunohistochemistry with the use of the H-Score system regardless of its cellular localisation or function. The definition of TTP in this study was based on the assumption that prognosis of newly diagnosed LC patients is a continuous function which depends on their tumour anatomic extent, as shown by stage. Hence, this definition of TTP was broader than the standard definition which is usually employed only in patients with advanced cancer who are followed up for tumour progression. It also included a category of early-stage patients whose tumour progression is usually expressed in terms of disease-free survival.

The most interesting finding in this study is certainly the relationship between TTP and p27 H-Score in LC patients. This relationship is not single-valued but has a very different character within the first eight-month interval after diagnosis and beyond that interval. This precludes the use of p27 H-Score as a single, independent prognostic factor for all LC patients. The latter finding can explain the difference in conclusions from previous studies regarding the prognostic value of p27 in LC patients. While most studies reported that p27 immunoreactivity was a positive prognostic factor for LC patients (13-19), some other studies reported inconsistent findings, especially with regard to non-squamous cell carcinoma (8-10).

The dual-valued relationship between p27 H-Score and TTP may reflect twofold effects of p27 on LC cell proliferation (20). Although p27 inhibits CDK, thus acting as a tumour suppressor gene, it also may promote stem cell expansion and function as a dominant oncogene (5). Tumour promoting effects of p27 are associated with cytoplasmatic mislocalisation and phosphorylation by phosphorylated kinases such as AKT at threonine 198, resulting in binding to RhoA. Activation of the RhoA/ROCK cascade increases cell motility and migration, a critical event in metastasis (6). As p27 RNA levels remain stable along cell cycle phases (2), p27 protein content or alternatively, cellular localisation remain the two important parameters to predict prognosis of LC patients. The present study did not analyse the ratio between nuclear and cytoplasmatic localisation, of p27 because of the high rate of subjective bias compared with p27 H-Score.

Molecular studies have identified multiple factors responsible for the modulation of tumour growth and prognosis of LC patients. It was previously shown that PIRH2 promotes ubiquitination of p27 (4). PIRH2 was a negative prognostic factor for all LC patients in the present study. Overexpression of PIRH2 was associated with short TTP in patients with metastasis or whose tumour progressed during the eight-month period after diagnosis, while p27 H-Score by itself was unable to predict prognosis in this category of patients. In patients whose tumours progressed after more than eight months, long TTP was associated with non-adenocarcinoma type of LC, high p27 H-Score and well-differentiated adenocarcinoma. Most patients who belong to this category usually have an operable, LC or otherwise, locally advanced tumours are adequately controlled with chemotherapy, radiotherapy, or both. For these patients, p27 H-Score may be considered a positive prognostic biomarker along with non-adenocarcinoma histology and favourable histological grade. In this context, it is interesting to note that in the (IALT) adjuvant study, patients with negative immunohistochemical expression of p27 benefited most from cisplatin-based adjuvant chemotherapy (21). Elevated p27 H-Score in patients with metastasis or patients whose tumour progressed during the eight-month period after diagnosis was probably associated with predominantly oncogenic properties of p27. More intense stain in patients who relapsed later was perhaps associated with predominantly tumour-suppressing effects. Despite the fact that the relationship between H-Score and duration of OS in patients with adenocarcinoma
Figure 1. Immunohistochemical studies for p27 (with diaminobenzidine, ×400). A: Lung adenocarcinoma negative for the p27 antibody (H-Score=0). B: Lung adenocarcinoma mildly-positive for the p27 antibody (H-Score=120). C: Lung adenocarcinoma strongly-positive for the p27 antibody (H-Score=240).

Figure 2. Distribution of p27 H-Score values for all lung cancer patients. The kernel curve appears in red.

Figure 3. Polynomial relationship between time-to-progression and p27 H-Score in patients with lung adenocarcinoma.

Figure 4. Probability of no progression according to PIRH2 status.
was dual-valued as well, OS duration was not associated with H-Score or any other parameter. In contrast to TTP, OS duration of was affected by additional factors such as the general condition of the patients and the treatment they received. These factors could conceal the prognostic effect of p27 H-Score concerning OS duration.

In summary, this model showed the difficulties in using p27 H-Score as a single prognostic factor in all LC groups. Since the relationship between p27 H-Score and TTP or duration of OS time was constructed as a polynomial model, the interpretation of p27 H-Score must be corroborated with other clinico-pathological parameters. Future studies in LC patients will probably need to consider biologic of parameters in addition to p27 for prognosis.

References


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