The Expression of Ki-67, but Not Proliferating Cell Nuclear Antigen, Predicts Poor Disease Free Survival in Patients with Adenocarcinoma of the Lung

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Abstract. Background: Ki-67 expression has been established as a predictive marker for recurrence in breast cancer, and proliferating cell nuclear antigen (PCNA) which is also a proliferation marker, is also herein discussed regarding its role in the prognosis of various types of cancer. However, no useful data are presently available regarding the biological significance of both molecules in lung cancer. Patients and Methods: Tumor specimens were collected from 183 consecutive patients who underwent a complete resection for lung adenocarcinoma from 2003 to 2007 in our Department. We analyzed the Ki-67 and PCNA expression levels in primary lung adenocarcinoma by immunohistochemistry. Results: Positive expression of Ki-67 and PCNA was identified in 41 (22.4%) and 149 (81.4%) patients, respectively. The positive expression of Ki-67 was identified in 14 (50.0%) out of 28 patients and 27 (18.1%) out of 155 patients with and without recurrence, respectively (p<0.001). PCNA expression was not correlated with recurrence. Positive expression of Ki-67 was associated with a poorer disease-free survival according to the survival analysis. A multivariate analysis also demonstrated that Ki-67 expression was independently associated with an increased risk of poor disease-free survival. Conclusion: Ki-67 may be a useful marker for predicting postoperative recurrence in patients with non-small cell lung cancer following complete resection.

Lung cancer is the leading cause of cancer-related death worldwide (1). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases, and the proportion of adenocarcinoma is notably increasing (2). Even when surgery, which is the major curative treatment, is performed, a certain population of patients nevertheless develops tumor recurrence (3). Therefore, it is necessary to identify those patients who might benefit the most from postoperative adjuvant chemotherapy in order to precisely select the patients who require additional treatment. As a result, it is important to evaluate the biological characteristics of NSCLC and identify the factors related to recurrence following surgery. However, no useful markers for predicting clinical recurrence exist at present.

Ki-67 is a marker of proliferation, and is expressed in all stages of the cell cycle except the G0 phase. Most studies have evaluated the prognostic significance of Ki-67 in frozen cryostat sections, since this cell cycle-related antigen was initially recognized by antibodies which could only bind on fresh frozen tissue (4). A new antibody, MIB1, has now become available, which can be used on formalin-fixed and paraffin-embedded sections (5). Proliferating cell nuclear antigen (PCNA) is a nuclear protein which is essential for DNA synthesis and it appears in the nucleus primarily during the S phase of the cell cycle (6, 7). Some studies have indicated that Ki-67 and PCNA were correlated with survival in patients with malignant melanoma and breast cancer (8, 9). However, the precise reason for the associated poor prognosis from a clinical standpoint remains unknown. There are currently no established biomarkers that have been correlated with the development of postoperative recurrence in lung cancer after surgery.

We therefore examined the relationship between the expression of both molecules and tumor aggressiveness with regard to the development of recurrence. If one or both of the molecules was found to be a marker of a poor prognosis, patients expected to have a poor prognosis could be selected for treatment with adjuvant chemotherapy.

Patients and Methods

Patients, clinical features, and follow-up. The Institutional Review Board approved this study, and informed consent for the use of the tumor specimens was obtained either from all the patients or from their legal guardians. Tumor samples were obtained from 296 patients with primary lung adenocarcinoma who had undergone a
surgical resection between 2003 and 2007 in our Department. Nine of these patients had stage IV disease, and 25 underwent an incomplete resection. The tumor samples from 79 patients were too small to evaluate by immunohistochemical (IHC) staining to determine the Ki-67 and PCNA status. As a result, 113 patients were excluded from further analysis. Therefore, 183 tumor specimens were evaluated.

The patients were followed-up every month within the first postoperative year and at approximately 2- to 4-month intervals thereafter. The evaluations included a physical examination, chest roentgenography, an analysis of blood chemistry, and measurements of tumor markers. Chest and abdominal computed tomography, brain magnetic resonance imaging, and a bone scintiscan were performed every 6 months for 3 years after surgery. Additional examinations were performed if any symptoms or signs of recurrence were detected. Follow-up was conducted for all 183 patients. The median follow-up period was 53.7 months. Twenty-seven (15.3%) patients received adjuvant chemotherapy as follows: carboplatin plus paclitaxel (n=18), carboplatin plus gemcitabine (n=7), and tegafur–uracil (UFT) (n=2) (10, 11). At the last follow-up, 144 patients were alive and free of cancer, while 11 patients had died of other causes without evidence of cancer, and 20 patients had died of cancer and 8 patients were alive with recurrent cancer. In total, 28 (15.3%) of the 183 patients demonstrated disease recurrence after surgery. The majority of the sites of tumor recurrence were hematogenous metastases. Twenty-five and six recurrence were hematogenous metastases. Twenty-five and six cases had hematogenous (9 brain, 10 lung, 5 bone, and 1 adrenal metastasis) and locoregional (4 lymph node metastasis and 2 pleural dissemination) recurrences, respectively. Two, one, and one patient had recurrent tumors in both the brain and bone, brain and adrenal gland, and bone and lymph nodes, respectively.

IHC staining of paraffin-embedded tumor samples. IHC staining was conducted using serial sections from the same paraffin-embedded blocks by previously described methods (12, 13). Briefly, all tissue specimens were formalin fixed and processed similarly, according to the standard histology practices. A 3-micron thick formalin-fixed, paraffin-embedded tissue section was prepared from each specimen. All specimens were stained with hematoxylin–eosin for the histological diagnosis. The sections were briefly immersed in citrate buffer [0.01 mol/l citric acid (pH 6.0)] and then were incubated twice for 10 min at 121°C in a high-pressure sterilization oven for antigen retrieval. They were then incubated with the Ki-67 antibody (MB1; Dako, Glostrup, Denmark) diluted at 1:100, and PCNA antibody (PC10; Dako) diluted at 1:500, in phosphate-buffered saline for 60 min at room temperature. Thereafter, IHC staining was performed by the labeled polymer method (Histofine Simple Stain MAX-PO kit, Nichirei, Tokyo, Japan) according to the manufacturer’s instructions (14). The positive controls for Ki-67 and PCNA were normal tonsil and colon cells, respectively. For negative control mouse IgG (Dako) was used instead of the primary antibody.

Evaluation of the stained specimens. Following the IHC detection of the protein expression in each specimen, the percentage of immunoreactive tumor cells in five <400 fields selected randomly on one slide was recorded, and then the final value for positive tumor cells was determined as the average of the positively immunostained cells. To evaluate any correlations with the clinicopathological characteristics, these protein expression scores were divided into positive or negative groups. The cut-off level for Ki-67 and PCNA staining was subdivided according to the percentages of nuclear staining as 20% and 50%, respectively (7, 15). The slides were independently examined by two of the investigators (S.O. and H.S.) who were blinded to the patients’ clinicopathological data. When a discrepancy was found between the two investigators, a consensus was reached via their simultaneous examination using a double-headed microscope.

Statistical analyses. Statistical significance was evaluated using the chi-square test or Fisher’s exact test. A multivariate logistic regression was used to evaluate independent associations. The Kaplan-Meier method was used to estimate the probability of survival, and survival differences were analyzed by the log-rank test. Disease free survival (DFS) was calculated from the date of treatment initiation to the date of documented progression. The terminal event of the OS analysis was death attributable to cancer or non-cancer causes. A multivariate analysis was then performed according to Cox’s proportional hazards model. The odds ratio (OR) and 95% confidence interval (95% CI) were calculated for each variable. Differences were considered to be statistically significant for p-values <0.05. The data were analyzed using the Stat View software package (Abacus Concepts, Inc., Berkeley, CA USA).

Results

Detection of the Ki-67/PCNA expression and correlation with clinicopathological factors. All of the patients were Japanese, consisting of 102 males and 81 females in this series, with a median age of 70 years (range 23–88 years). The tumor stage was classified according to the TNM Classification for Lung Cancer (16). According to the pathological stage, 106 patients had tumors of stage IA, 39 of IB, 13 of IIA, 6 of IIB, 16 of IIIA and 3 of stage IIIB. Ki-67 and PCNA expression were identified in 41 (22.4%) and 149 (81.4%) patients, respectively. A significant association between Ki-67 expression and gender, pathological stage, T status, N status, smoking history, tumor grade, and the tumor marker carcinoembryonic antigen (CEA) was identified. On the other hand, a significant association was only observed between the PCNA expression and the patient age and T status. No significant association was observed between Ki-67 and the PCNA expression (Table I). We also examined the correlation between Ki-67 and PCNA using the practical positive percentage rate. However, this also failed to present any significant correlation.

Influence of Ki-67/PCNA expression on postoperative recurrence. The positive expression of Ki-67 was identified in 14 (50.0%) out of 28 patients and 27 (17.4%) out of 155 patients with and without recurrence, respectively (p<0.001) (Table II). Positive expression of PCNA was identified in 23 (82.18%) and 126 (81.3%) of the patients with and without recurrence, respectively (p=0.915). Therefore, the positive expression of Ki-67, but not PCNA, was significantly correlated with postoperative recurrence (Table II).
Influence of Ki-67/PCNA expression on DFS. The 5-year DFS rate in patients negative and positive for Ki-67 expression was 82.2% and 48.0%, respectively ($p<0.001$) (Figure 1A). The 5-year OS rate in those negative and positive for Ki-67 expression was 86.6% and 54.2%, respectively ($p<0.001$) (Figure 1B). PCNA expression did not effect the DFS and OS (Figure 1C and D).

Ki-67, but not PCNA, was also found to be associated with a poorer DFS according to the univariate survival analysis ($p<0.001$) (Table III). A multivariate survival analysis also demonstrated that Ki-67 expression, but not the PCNA expression, to be independently associated with an increased risk for a poor DFS ($p=0.016$) (Table IV).

Discussion

This investigation was unique for three reasons. Firstly, were surgical specimens used after complete resection. Secondly, it was limited to the analysis of adenocarcinoma, which is relatively homogeneous. Thirdly, the method is based on simple IHC staining, which has the advantage of maintaining the morphology of the tissue, and minimizing sampling error (5).

We found significant correlations between the Ki-67 expression and recurrence in lung adenocarcinoma. Ki-67 expression was also associated with a poorer overall survival. This finding suggests that Ki-67 expression might be a suitable biomarker to identify candidate patients who would benefit from additional treatment.
benefit most from adjuvant chemotherapy for adenocarcinoma following a complete resection. On the other hand, PCNA expression was not found to significantly correlate with recurrence, and no significant correlation was seen between the PCNA and Ki-67 expression. Some researchers have shown that Ki-67 had a significant positive correlation with PCNA, and that Ki-67 and PCNA were correlated with survival in malignant melanoma (8), and breast cancer (9). The discrepancy between these studies and our present study might be due to the analyzed number of specimens, homogeneity of the specimens examined, or the target organ.

Both Ki-67 and PCNA are expressed in proliferating cells, but there are important differences among the two. Ki-67 starts being expressed in the mid-G1 phase, is expressed through S and G2, and reaches its peak in M phase, and it is very rapidly degraded at the end of M phase (17).
Table III. Univariate analysis using a proportional hazard model for the disease-free survival.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Characteristics</th>
<th>95% CI</th>
<th>HR</th>
<th>p-value</th>
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<tr>
<td>Gender</td>
<td>Male</td>
<td>1.247-4.950</td>
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<td>0.010</td>
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<td>Age (years)</td>
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<td>0.907-3.155</td>
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<td>Smoking history</td>
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<td>T-Status</td>
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<td>2.192-6.623</td>
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<td>&lt;0.001</td>
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<td>PCNA</td>
<td>Negative</td>
<td>0.827-3.275</td>
<td>1.645</td>
<td>0.156</td>
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CI: Confidence interval, HR: hazard ratio, F/C: former + current, PCNA: proliferating cell nuclear antigen.

Table IV. Multivariate analysis using a proportional hazard model for the disease-free survival.

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<td>N-Status</td>
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<td>Ki-67</td>
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PCNA: Proliferating cell nuclear antigen.

Conclusion

The current results indicate that the Ki-67 expression may be a useful marker for predicting postoperative recurrence in patients with lung adenocarcinoma following surgery. Further investigations will be necessary to examine the optimal method of detecting Ki-67 expression, for validation of the method, and for determining the efficacy of adjuvant chemotherapy.

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References


