A Risk-Stratification Model of Non-Small Cell Lung Cancers Using Cyclin E, Ki-67, and ras p21: Different Roles of G1 Cyclins in Cell Proliferation and Prognosis

Hirotoshi Dosaka-Akita, Fumihiro Hommura, Takayuki Mishina, Shigeaki Ogura, Michio Shimizu, Hiroyuki Katoh, and Yoshikazu Kawakami

INTRODUCTION

Lung cancer is one of the leading causes of cancer death throughout the world. Whereas the management and treatment of NSCLCs have improved, there is no evidence to suggest that therapeutic advances have resulted in a marked increase in survival rates, and the overall 5-year survival rate remains <15% (1). The clinical observations that patients having NSCLCs in comparable stages may have different clinical courses and may respond differently to similar treatments have yet to be fully understood. Clearly, a more sophisticated understanding of the pathogenesis and biology of these tumors (2, 3) could provide useful information for predicting clinical outcome and individualizing treatment (4–6). It could also provide important information for identifying molecular targets of the treatment (7–9). If it is possible to develop a reliable method to estimate a patient’s risk of recurrence after resection of p-stage I NSCLC by integrating several prognostic factors, it will facilitate the development of rational post-resection treatment strategies based on the estimated risk of recurrence.

Altered regulation of the cell cycle is a hallmark of human cancers (10, 11). The cell cycle is governed by cdks, the activity of which is regulated by the binding of positive effectors, the cyclins (12, 13), and by negative regulators, the cdk-inhibitors (14, 15). The cdks integrate mitogenic and growth-inhibitory signals and coordinate cell cycle transitions (12, 13). Progression through the G1 phase of the cell cycle is dependent upon the activity of G1 cyclins, which include the D-type cyclins and cyclin E. The D-type cyclins reach maximal levels of expression and form functional kinase complexes with cdk4 or cdk6 during the mid-G1 phase (16–21), whereas cyclin E is expressed and associated with cdk2 in an active complex near the G1-S boundary (22, 23). G1 cyclin expression seems to be specific for tumor cells, to represent a true tumor-associated abnormality, and to be a potential prognostic marker of various types of cancer (24–30). However, there are no studies that have analyzed cyclin D1 and cyclin E with regard to cell proliferation and clinical outcome in a single cohort of NSCLCs.

Previously, we have reported alterations of regulator molecules of the G1-S transition, including p53, RB, p16INK4A, and p27KIP1, and their biological and prognostic importance in resected NSCLCs (31–33). Moreover, we have recently demonstrated the following by immunohistochemistry: (a) that positive cyclin D1 expression is associated with longer survival and tends to be a favorable prognostic factor in a small cohort of resected NSCLCs, including 77 p-stage I-IIa tumors (36); and (b) that high-level cyclin E expression is a significant and independent unfavorable prognostic factor in another cohort of NSCLCs, including 103 p-stage I tumors (37).

In the present study, we characterized expression of both G1 cyclins to analyze (a) their expression in relation to the Ki-67 cell growth fraction to determine the implication of both G1 cyclins in the cell cycle regulation of NSCLCs; and (b) their prognostic importance in a larger cohort of resected NSCLCs compared with the previous cohort used for cyclin D1 (the same one used for cyclin E) from a single institution. Furthermore, we attempted to stratify patients with p-stage I NSCLCs into different survival groups by a combination of biological factors, including cyclin E, Ki-67, and ras p21, which we have previously shown to be independent prognostic factors among 10 biological factors in p-stage I NSCLCs (31, 35, 37).

MATERIALS AND METHODS

Tumor Specimens and Survival Data. The present cohort of NSCLCs is the same as the one that we used previously for the analysis of the expression of cyclin E alone (37). Primary tumor specimens from 217 NSCLCs were consecutively obtained by surgery from the Hokkaido University Medical Hospital during 1976 and 1994. The patients with NSCLCs consisted of 147 men and 70 women (average age at diagnosis, 63.3 years). The histological classification of the tumor specimens was based on WHO criteria (38). The specimens included 92 squamous cell carcinomas, 107 adenocarcinomas, 9 large cell carcinomas, and 9 adenosquamous cell carcinomas. They represented the following stage distribution: stage I, 119 stage I, 18 stage II, 72 stage IIIa, 1 stage IIIb, and 7 stage IV tumors. The postsurgical pathological Tumor-Node-Metastasis stage was determined ac-
Table 1 Relationship between cyclins D1 and E and Ki-67 LI in resected NSCLCs

<table>
<thead>
<tr>
<th>Cyclin</th>
<th>No.</th>
<th>Ki-67 LI</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>D1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>57</td>
<td>41.6 ± 24.9</td>
<td>0.1</td>
</tr>
<tr>
<td>(−)</td>
<td>158</td>
<td>35.3 ± 28.0</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>115</td>
<td>45.9 ± 25.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Low</td>
<td>99</td>
<td>26.6 ± 25.9</td>
<td></td>
</tr>
</tbody>
</table>

*Cyclin D1 (+), positive nuclear staining for cyclin D1 in at least several malignant cells. Cyclin D1 (−), no nuclear staining for cyclin D1 in any malignant cells.

**High-level cyclin E expression, cyclin E LI ≥30%. Low-level cyclin E expression, cyclin E LI <30%.

According to the guidelines of the American Joint Committee on Cancer (39). A total of 176 tumors were potentially curatively resected. Of the 176 patients, survival was analyzed for the 151 patients who met the following criteria: (a) survived for >3 months after surgery; (b) did not die of causes other than lung cancer within 5 years after surgery; and (c) were followed for >3 years after surgery (for patients who remained alive). Fourteen patients who did not meet the above criteria (six died within 3 months after surgery, and eight died of causes other than lung cancer within 5 years) were excluded from the survival analysis. Eleven patients for whom no survival records after surgery were obtained were also excluded from the survival analysis. One hundred ten patients received combination chemotherapy as postsurgical treatment. Radiation therapy was not performed before or after surgery for any patients. Because all of the patients were coded, they could not be individually identified.

Immunohistochemistry. Expression of cyclin D1, cyclin E, and Ki-67 was analyzed by immunohistochemistry. For the cyclin E and Ki-67 staining, the slides and results that were reported previously (35, 37) were used for the present study. The methods for the staining of cyclin D1, cyclin E, and Ki-67 of resected tumors have been described previously (35–37). The labeled streptavidin biotin method was used on 4-μm sections of formalin-fixed, paraffin-embedded tissues after deparaffinization and antigen retrieval. The primary antibodies used were a mouse monoclonal antihuman cyclin D1 antibody, DCS-6 (Oncogene Science, Inc., Manhasset, NY), a mouse monoclonal antihuman cyclin E antibody, HE12 (PharMingen, San Diego, CA), and a mouse monoclonal MIB-1 antibody (Immunotech, Marseilles, France). Methyl green was used as the counterstain.

Observing one whole specimen from each tumor, tumors were regarded as cyclin D1-positive (+) if at least several malignant cells had nuclear staining for cyclin D1. Tumors were scored as cyclin D1-negative (−) if no malignant cells had any nuclear staining for cyclin D1 (36).

For cyclin E and Ki-67, the LI (%) was defined as the percentage of tumor cells displaying nuclear immunoreactivity, and was calculated by counting the number of nuclear-stained tumor cells in 1000 tumor cells in each section. A single representative tissue section from each tumor was surveyed microscopically at ×100 for at least two or three areas with the highest intensity of positive tumor cells. Cell counts were performed at ×400 in at least five fields in these areas using a Videomicrometer (Model VM-30; Olympus, Tokyo, Japan) equipped with a light microscope. The cyclin E and Ki-67 LIs were reliably and reproducibly obtained using this Videomicrometer system. All immunohistochemical studies were done without knowledge of the clinical data.

Statistical Analysis. The associations between cyclin expression and categorical variables were analyzed by the χ² test or Fisher's exact test as appropriate. The associations between cyclin expression and age were analyzed by Student’s t test. The survival curves were estimated using the Kaplan-Meier method, and differences in survival distributions were evaluated by the generalized Wilcoxon test. Cox’s proportional hazards modeling of factors potentially related to survival was performed to identify which factors might have a significant influence on survival. The significance level chosen was P < 0.05, and all tests were two-sided.

RESULTS

Positive cyclin D1 expression was found in 59 (27%) of 218 NSCLCs in the present study. High-level cyclin E expression (a cyclin E LI ≥30%) was found in 115 (53%) of 217 NSCLCs, as reported previously (37). The association between cyclin D1 expression and the Ki-67 LI and between cyclin E expression and the Ki-67 LI were analyzed in 215 and 214 tumors, respectively (Table 1). Positive or negative cyclin D1 expression was not associated with the Ki-67 LI (P = 0.1). Tumors having high-level cyclin E expression showed a higher Ki-67 LI than tumors having low-level cyclin E expression (P < 0.001). The cyclin E LI was significantly associated with the Ki-67 LI (r = 0.45; 95% CI, 0.33–0.55; P < 0.001). In addition, positive or negative cyclin D1 expression was not correlated with high- or low-level cyclin E expression or with cyclin E LI (data not shown).

The status of cyclin D1 was not correlated with clinical and clinicopathological characteristics of tumors, including age, gender, smoking, histology, pathological Tumor-Node-Metastasis stage classification, and p-stage (data not shown). High-level cyclin E expression was significantly more prevalently found in tumors from men than in those from women, in tumors from smokers compared with nonsmokers, in squamous cell carcinomas compared with nonsquamous cell carcinomas, and in pT2–4 tumors compared with pT1 tumors by the χ² test. A significant association of high-level cyclin E expression with squamous cell carcinoma was found by multivariate logistic regression analysis (37).
We next analyzed the relationship between the expression of both cyclins and survival in p-stage I NSCLCs. There was no significant difference in survival between patients with tumors having positive and negative cyclin D1 expression in 104 p-stage I NSCLCs (5-year survival rates, 77% and 62%, respectively; \( P = 0.2 \); Fig. 1A). The status of cyclin D1 expression was not a prognostic factor \( (P = 0.2) \).

On the other hand, patients with tumors having high-level cyclin E expression survived a significantly shorter time than patients with tumors having low-level cyclin E expression in 103 p-stage I NSCLCs (5-year survival rates, 57% and 81%, respectively; \( P = 0.007 \); Fig. 1B) as reported previously \((37)\). High-level cyclin E expression was a significant and independent unfavorable prognostic factor \( (\text{hazards ratio} = 2.09; 95\% \text{ CI}, 1.06 – 4.13; P = 0.03) \).

Previously, we have studied the prognostic importance of various biological factors, including \( \text{ras} \), \( p21 \), \( p53 \), \( RB \), \( p16 \), \( p27 \), cyclin D1, cyclin E, Ki-67, Bcl-2, and gelsolin, by immunohistochemistry in resected NSCLCs \((31, 33–37, 40 – 42)\). Among these 10 factors, cyclin E, Ki-67, and \( \text{ras} \) were independently significant prognostic factors in p-stage I NSCLCs. These three prognostic factors were used to design a model to predict an individual patient’s risk of cancer death. For this model, cyclin E expression was scored as high \((\text{cyclin E LI} \geq 30\%) \) and low \((\text{cyclin E LI} < 30\%) \); Ki-67 expression was scored as high \((\text{Ki-67 LI} \geq 30\%) \) and low \((\text{Ki-67 LI} < 30\%) \); and \( \text{ras} \) expression was scored as positive or negative as reported previously \((31, 35, 37)\). The relative risk of cancer death in each patient was characterized by summing the score of each prognostic factor, as follows: (a) score 1 (altered) for high-level cyclin E expression, high-level Ki-67 expression, and positive \( \text{ras} \) expression, respectively; (b) and score 0 (unaltered) for low-level cyclin E expression, low-level Ki-67 expression, and negative \( \text{ras} \) expression, respectively (Fig. 2). Four groups of patients with markedly different survivals were identified in p-stage I NSCLCs, as follows: (a) patients having a total score of 0 with 96% 5-year survival; (b) those having a total score of 1 with 88% 5-year survival; (c) those having a total score of 2 with 53% 5-year survival; and (d) those having a total score of 3 with 41% 5-year survival \( (P < 0.001; \text{Fig. 3}) \).

A higher total score was significantly more prevalently found in squamous cell carcinomas compared with nonsquamous cell carcinomas, and in moderately and poorly differentiated tumors compared with well-differentiated tumors \((Table 2)\). This risk-stratification model of NSCLCs, using cyclin E, Ki-67, and \( \text{ras} \), was a significant and independent prognostic factor \( (\text{hazard ratio} = 7.94; P = 0.001) \), whereas histology and differentiation of tumors were not \((Table 3)\).

Fig. 2. Immunohistochemical staining patterns for cyclin E \((A) \) and \( B \), Ki-67 \((C) \) and \( D \), and \( \text{ras} \) \((E) \) and \( F \) in NSCLCs. The left column of panels \((A, C, \text{and } E) \) show scores of 0 for expression of each protein: low-level cyclin E expression \((A) \), low-level Ki-67 expression \((C) \), and negative \( \text{ras} \) \( p21 \) expression \((E) \), respectively. The right column of panels \((B, D, \text{and } F) \) show scores of 1 for expression of each protein: high-level cyclin E expression \((B) \), high-level Ki-67 expression \((D) \), and positive \( \text{ras} \) \( p21 \) expression \((F) \), respectively. \( \text{Bar} = 20 \mu m. \)

Fig. 3. Kaplan-Meier survival curves of patients with p-stage I NSCLCs. Survival curves are stratified according to a total score of prognostic factors, including cyclin E, Ki-67, and \( \text{ras} p21 \).
DISCUSSION

The present study demonstrated different roles of cyclin D1 and cyclin E in cell proliferation and clinical outcome of NSCLCs in a cohort of p-stage I disease from a single institution. Cyclin E expression was positively associated with the cell growth fraction determined by the Ki-67 LI and was an independent unfavorable prognostic factor, whereas cyclin D1 expression was not associated with the Ki-67 LI or clinical outcome. More importantly, a combination of three independent prognostic factors, including cyclin E, Ki-67, and ras p21, stratified patients with p-stage I NSCLCs into markedly different survival groups.

Both cyclin E and cyclin D1 are involved in the regulation of the G1-S transition of the cell cycle (12, 13). However, high expression of these proteins can cause different molecular and cellular biological phenotypes of cancer cells; cyclin E can induce chromosome instability (43), which is involved in the development and progression of tumors; cyclin D1 plays a role in the control of apoptosis (44) and growth suppression (45). Such different and multifunctional properties of these cyclins may contribute to their different effects on cell proliferation and clinical outcome of NSCLCs.

A few studies support the possibility that multiple markers might be more informative than any single markers for the prediction of clinical outcome of p-stage I NSCLCs (4–6). We attempted to construct a multivariate model predicting the risk of recurrence and cancer death in p-stage I NSCLCs. Among the 10 biological factors that we studied previously, three were independent prognostic factors for p-stage I disease. The presence or absence of alterations of these three factors, including cyclin E, Ki-67, and ras p21, were scored and summed. The obtained total score for each tumor clearly stratified the patients with p-stage I NSCLC into markedly different survival groups, with 5-year survival rates that ranged from 96% for patients with no factors altered to 41% for patients with all three factors altered.

In conclusion, we showed different roles of cyclin D1 and cyclin E in cell proliferation and clinical outcome of NSCLCs. Cyclin E expression is involved in the cell growth and prognosis of NSCLCs, suggesting that it may have great value in identifying NSCLC patients at high risk of early disease recurrence after surgery, and that it may be a good candidate as a molecular target for the treatment of NSCLCs (7–9). Moreover, a combination of three independent prognostic factors, including cyclin E, Ki-67, and ras p21, stratified NSCLC patients into markedly different survival groups. If the current findings are confirmed in prospective studies with p-stage I NSCLCs, this combination of prognostic factors can be used to identify patients at high risk of early disease recurrence and cancer death after surgery, and to select patients who will benefit from intensive adjuvant therapy. We are currently planning such a prospective study for p-stage I NSCLCs, stratifying patients into different groups according to this combination of prognostic factors. Those classified as having a low risk of recurrence would receive surgical resection alone, whereas high-risk patients would be randomized to receive surgical resection alone or resection followed by adjuvant chemotherapy (4).

**REFERENCES**

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