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

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ABSTRACT

A large number of biological factors that seem to have important prognostic significance have been identified in non-small cell lung cancers (NSCLCs). In the present study, we have characterized expression of cyclin D1 and cyclin E in a cohort of 217 resected NSCLCs from a single institution by immunohistochemistry to analyze their expression in relation to the growth fraction determined by Ki-67 and to prognosis, and then we have constructed a risk-stratification model of cancer death by multiple biological factors in p-stage I NSCLCs. The cyclin E labeling index (LI) was significantly associated with the Ki-67 LI \((r = 0.45; \ P < 0.001)\). Tumors having high-level cyclin E expression (cyclin E LI \(\geq 30\%\)) showed a significantly higher Ki-67 LI than tumors having low-level cyclin E expression (cyclin E LI \(< 30\%\); \(\ P < 0.001\)), whereas positive or negative cyclin D1 expression was not associated with the Ki-67 LI \(\ (P = 0.1)\). Cyclin E expression was a significant and independent unfavorable prognostic factor (hazards ratio = 2.09; \(\ P = 0.03\)), as reported previously (Clin. Cancer Res., 6: 11–16, 2000), whereas cyclin D1 expression was not. These findings indicate different roles of cyclin D1 and cyclin E in cell proliferation and in the prognosis of NSCLCs. Furthermore, we stratified this cohort of p-stage I NSCLCs into different survival groups by using biological factors, including cyclin E, Ki-67, and \(\text{ras}\) p21, which previously we have found to be independent prognostic factors among 10 factors studied in p-stage I NSCLCs. Four groups of patients with markedly different survivals were identified with 5-year survival rates that ranged from 96% for patients with no factors altered to 41% for patients with all three factors altered \((P < 0.001)\). This combination of biological factors was a significant and independent prognostic factor (hazards ratio = 7.94; \(\ P = 0.001\)).

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 postresection 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 G\(_1\) 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-G\(_1\) phase \((16–21)\), whereas cyclin E is expressed and associated with cdk2 in an active complex near the G\(_1\)-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 G\(_1\)-S transition, including p53, RB, p16\(^{INK4A}\), and p27\(^{KIP1}\), and their biological and prognostic importance in resected NSCLCs \((31–35)\). 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 \((1)\) 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 \(\text{ras}\) 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 \((\text{average age at diagnosis, 63.3 years})\). The histological classification of the tumor specimens was based on WHO criteria \((38)\), and the specimens included 92 squamous cell carcinomas, 107 adenocarcinomas, 9 large cell carcinomas, and 9 adenosquamous cell carcinomas. They represented 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-

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\* The abbreviations used are: NSCLC, non-small cell lung cancer; cdk, cyclin-dependent kinase; LI, labeling index; CI, confidence interval; p-stage, pathologic stage.
Table 1 Relationship between cyclins D1 and E and Ki-67 LI in resected NSCLCs

<table>
<thead>
<tr>
<th>Cyclin D1</th>
<th>Ki-67 LI</th>
<th>P</th>
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<tbody>
<tr>
<td>(+)</td>
<td>57 (27%)</td>
<td>41.6 ± 24.9</td>
</tr>
<tr>
<td>(−)</td>
<td>158 (73%)</td>
<td>35.3 ± 28.0</td>
</tr>
<tr>
<td>Cyclin E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highb</td>
<td>115 (54%)</td>
<td>45.9 ± 25.5</td>
</tr>
<tr>
<td>Lowb</td>
<td>99 (46%)</td>
<td>26.6 ± 25.9</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%.

Cyclin E LI was significantly associated with the Ki-67 LI (r = 0.45; 95% CI, 0.33–0.55; P < 0.001).

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).

![Fig. 1. Kaplan-Meier survival curves of patients with p-stage I NSCLCs. Survival curves are stratified by negative (−) and positive (+) cyclin D1 expression (A), and by low- and high-level cyclin E expression (B).](image-url)
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 (hazards ratio \( = 2.09 \); 95% 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\(^{INK4A} \), p27\(^{KIP1} \), 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 \( \text{ras} \) p21 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 (cyclin E LI \( \geq 30\% \)) and low (cyclin E LI \(< 30\% \)), Ki-67 expression was scored as high (Ki-67 LI \( \geq 30\% \)) and low (Ki-67 LI \(< 30\% \)), and \( \text{ras} \) p21 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 \( \text{ras} \) p21 expression, respectively; (b) and score 0 (unaltered) for low-level cyclin E expression, low-level Ki-67 expression, and negative \( \text{ras} \) p21 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 (\( P < 0.001 \); 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 \( \text{ras} \) p21, was a significant and independent prognostic factor (hazard ratio \( = 7.94 \); \( P = 0.001 \)), whereas histology and differentiation of tumors were not (Table 3).

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 with 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).

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**Table 3** Cox’s proportional hazards model analysis of prognostic factors in patients with p-stage I NSCLCs

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>A. Univariate analysis of potential prognostic factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>0.48</td>
<td>0.23–1.00</td>
<td>0.051</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>1.24</td>
<td>0.72–2.15</td>
<td>0.4</td>
</tr>
<tr>
<td>Histology (nonsquamous/squamous)</td>
<td>1.70</td>
<td>0.93–3.13</td>
<td>0.09</td>
</tr>
<tr>
<td>Differentiation (moderate, poor/ well)</td>
<td>0.73</td>
<td>0.34–1.60</td>
<td>0.4</td>
</tr>
<tr>
<td>pT classification</td>
<td>1.00</td>
<td>0.54–1.86</td>
<td>1.0</td>
</tr>
<tr>
<td>Cyclin E/Ki-67/ras p21 (Total score: 0/1/2/3)</td>
<td>8.55</td>
<td>2.47–29.41</td>
<td>0.0007</td>
</tr>
<tr>
<td>B. Multivariate analysis of prognostic factors a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>0.60</td>
<td>0.25–1.45</td>
<td>0.3</td>
</tr>
<tr>
<td>Histology (nonsquamous/squamous)</td>
<td>0.93</td>
<td>0.44–1.94</td>
<td>0.8</td>
</tr>
<tr>
<td>Cyclin E/Ki-67/ras p21 (Total score: 0/1/2/3)</td>
<td>7.94</td>
<td>2.24–27.78</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Potential prognostic factors selected from Table 3A.

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**REFERENCES**


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