Abstract

We analyzed 66 non-small cell lung cancer cell lines for mutations at codons 12, 13, and 61 of all three ras genes and correlated the findings with patient survival. We used designed restriction fragment-length polymorphisms to detect mutations after amplification of ras-specific sequences by the polymerase chain reaction. We found 19 mutations of ras genes (29%), and 11 of these 19 (58%) were at codon 12 of the K-ras gene. By univariate analysis, the presence of any ras mutation in cell lines from patients who received curative intent treatment was associated with a shorter survival ($P_1 = 0.002$). For patients who received only palliative treatment, detection of K-ras mutations at codon 12 was associated with a shortened survival ($P_2 = 0.0103$), but this analysis was not statistically significant for the group with any ras mutation ($P_1 = 0.093$). The Cox proportional hazards model also predicted a higher risk for patients with any type of ras mutations. We conclude that ras mutations, present in a subset of non-small cell lung cancers, are independently associated with the shortened survival of patients, irrespective of treatment intent.

Introduction

Lung cancer is the leading cause of cancer death in the United States, with almost 90% of the patients dying from the disease within 2 years of diagnosis (1). NSCLCs² comprise about 75% of lung cancer and consist of adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and rare types (1). The small percentage of patients who are cured are generally from among the patients (one-third) who present with localized disease which is totally resected by surgery (1). The remaining patients with regionally advanced or distant metastatic disease are treated with radiation therapy, combination chemotherapy, or supportive care (1).

The ras gene family consists of K-, H-, and N-ras genes, which code for similar membrane-bound $M$, 21,000 proteins, which have an important role in the cellular signal transduction pathway (2). The ras genes can be activated by point mutations occurring at codon 12, 13, or 61, resulting in an oncogenic potential (2). The activated ras genes are the most frequently found oncogenes in a variety of human cancers (3). In lung cancers, activated ras genes, especially the K-ras gene, are found in about 20–30% of resected NSCLC specimens (4–6). Slebos et al. (7) have recently shown that K-ras mutations are associated with a poor prognosis in patients with adenocarcinoma of the lung who undergo curative resection.

We previously examined the incidence of ras mutations in 103 lung cancer cell lines and found that (a) 35% of the NSCLC lines including all histological subtypes had ras mutations; (b) sex, prior treatment status, disease extent, and in vitro culture time did not affect the incidence of ras mutations; and (c) none of 37 small cell lung cancer cell lines had any ras mutations (8). In this study, we examined a panel of NSCLC cell lines including all histological types of NSCLC established from patients with all stages including advanced disease. As a result, we were able to confirm the observation of Slebos et al. (7) regarding the negative prognostic correlation of ras mutations in early-stage NSCLC. In addition, we report, for the first time, that the detection of a ras mutation in advanced-stage patients is also associated with shortened survival and may define a subset of NSCLC patients especially appropriate for investigational therapeutic approaches.

Materials and Methods

Patients and Cell Lines. Between August 1976 and May 1990, viable tumor tissues from patients with pathologically proven primary or metastatic NSCLC were obtained as part of standard diagnostic, staging, or therapeutic procedures following appropriate informed consent (9). Approximately 100 cell lines were established during that time using a previously described culture technique (10). The criteria for inclusion of the patients in the present study were availability of a cell line and clinical data regarding treatment intent and survival. Accordingly, 66 patients (40 adenocarcinomas, 5 squamous cell carcinomas, 12 large cell carcinomas, 5 carcinoids, and 4 other types) were included for the present analysis. The patients were divided into 2 groups according to the treatment they received. The potentially curative group ($n = 21$) consisted of patients with tumors confined to one hemithorax (stages I, II, and selected, favorable IIIa) by the new American Joint Committee staging system; Ref. 11 who underwent surgery (19 patients) or who received radiotherapy with curative intent (2 patients). The palliative group (45 patients) consisted of those with locally advanced or metastatic disease (stages IIIb and IV) who received chemotherapy and/or palliative radiation or supportive treatment.

Detection of ras Mutations. Point mutations of all three ras genes at codons 12, 13, and 61 were detected by a nonradiative technique utilizing designed restriction fragment-length polymorphisms as previously described (8, 12, 13). Briefly, a mismatched primer was used in a polymerase chain reaction of genomic DNA so that the combination of the wild-type sequence of a given codon and the mismatch introduced by the polymerase chain reaction primer would be recognized by an appropriate restriction enzyme. A specific cleavage was detected byagarose gel electrophoresis following the restriction enzyme digestion. Subsequent to this initial screening, all the base substitutions were identified and confirmed by other restriction fragment-length polymorphism analyses in which a specific mutant sequence was cut or by direct sequencing (8).

Statistical Analysis. The Kaplan-Meier method was used to estimate the probability of survival as a function of time (14), and the Mantel-Haenszel method was used to assess the significance of the difference between pairs of survival probabilities (15). The Cox proportional hazards modeling technique was used to identify which independent

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factors had a jointly significant influence on overall survival (16). All reported \( P \) values are two sided (denoted by \( P_2 \)).

Results
Detection of Point Mutations in \( ras \) Oncogenes. We detected 19 \( ras \) mutations in 66 NSCLC cell lines (29%). Of 19 mutations, 11 were at codon 12 of \( K-ras \) (6 were GGT to TGT; 2 were GGT to GTT; 2 were GGT to CGT; and one was GGT to GCT); 4 were at codon 13 of \( K-ras \) (3 were GGC to TGC and one was GGC to GAC); one was at codon 61 of \( K-ras \) (CAA to CAT); one was at codon 61 of \( H-ras \) (CAG to CTG); and 2 were at codon 61 of \( N-ras \) (one was CAA to AAA and one was CAA to CGA). Thus 16 of 19 mutations (84%) occurred in the \( K-ras \) gene, of which 69% were at codon 12.

Mutations were present in adenocarcinomas (8 of 40 or 20%) and other types of NSCLC (11 of 26 or 42%).

Selection of Starting Point for Measurement of Patient Survival. In this study, tumor material was available at varying time intervals relative to the patients' initial diagnosis. We first considered which time point, date of diagnosis, or date of tissue acquisition was more appropriate as the initial time point in measurement of patient survival. We constructed Cox models using each of these starting points. In each case, the final model contained identical variables (discussed later). Because it is possible that mutations may have occurred after the date of diagnosis but prior to tissue acquisition, we selected the latter date as the more appropriate.

Univariate Analyses. The variables listed in Table 1 included all standard prognostic factors that were evaluated in univariate analyses. Type of treatment, interval between diagnosis and tissue acquisition, prior treatment status, performance status, and \( ras \) mutation at codon 12 of \( K-ras \) were significant prognostic factors (Table 1).

The Kaplan-Meier survival curves demonstrated that when all the patients were considered, those with any \( ras \) mutation or a \( K-ras \) 12 mutation survived for a shorter period of time, although any \( ras \) mutation had a borderline significance (Fig. 1). When a comparison was made within groups that received curative or palliative treatment, the effect of \( ras \) mutations on survival became more prominent (Fig. 2). The difference between patients with any \( ras \) mutation and those without mutation in the palliative treatment group did not reach statistical significance.

Cox Proportional Hazards Model. Data were analyzed using the Cox proportional hazards model. The universe of variables from which the Cox models were constructed initially included all the variables used in univariate analyses as shown in Table 1. We have developed 4 models (Table 2). In models 1 and 2, \( ras \) mutations, curative procedure, and the interval between diagnosis and tissue acquisition were important prognostic factors. Elimination of this tissue diagnosis interval did not change the effect of \( ras \) mutations and curative treatment, as in models 3 and 4. Therefore, patients whose cell lines had any \( ras \) mutations had a significantly greater risk of earlier death than those without mutations. The substitution of any \( ras \) mutation by mutation at \( K-ras \) codon 12 slightly improved this model.

Discussion
Slebos et al. (7) reported that the presence of a \( K-ras \) mutation at codon 12 is a negative prognostic factor among patients with adenocarcinoma of the lung who underwent radical resection. In our study, we analyzed a broader subset of patients in terms of disease extent and treatment. We confirmed the Dutch finding and, furthermore, were able to show that the presence of any \( ras \) mutation is an important prognostic factor among those who received only palliative treatment. We have previously presented evidence that support the concept that \( ras \) mutations found in cell lines arose in vivo and not as a function of in vitro propagation (8). Our data indicated that \( K-ras \) mutations at codon 12 might be more important than those at
other codons by multi- as well as univariate analyses. However, we cannot conclusively prove this point because of limited numbers for each subset in this analysis. We found ten different ras mutations in 19 cell lines. These different mutations may have varying effects on patient survival because it has been shown that the transforming ability of the H-ras gene activated at codon 61 depends on base substitution (17). However, because of the limited number for each subset, we could not analyze this point.

The mechanism by which a ras mutation affects the clinical outcome is unknown. ras mutations confer invasive or metastatic potential under certain experimental conditions (18, 19). However, the similar incidence of ras mutations in primary lung tumor and in regionally advanced or metastatic tumors (8) is at odds with this supposition. Transfection of an activated ras gene has been reported to induce resistance to cis-diaminedichloroplatinum(II) or resistance to ionizing radiation (20, 21), and both of these modalities are frequently used in the care of NSCLC patients. Direct evaluation of chemosensitivity to 6 agents including cis-diaminedichloroplatinum(II) demonstrated that chemoresistance was independent of ras mutations in our NSCLC cell lines. Stevenson et al. (9) reported that the ability to establish a cell line from a tumor tissue is a new, independent prognostic factor for the survival of patients with NSCLC. However, it is unlikely that cell line establishment is closely related to ras status at least in adenocarcinomas, because the incidence of ras mutations in our adenocarcinoma cell lines was not higher than that in tumor specimens (5, 6, 8).

In other types of NSCLC, however, the incidence of ras mutations in cell lines was considerably higher than that in tumor specimens (5, 6, 8). Therefore, ras mutations may aid in vitro growth in these types of NSCLC.

Alterations of some oncogenes including ras genes have been

Table 2 Cox proportional hazards model for factors associated with survival of NSCLC patients

| Model | Variable | P value | Relative risk | 95% confidence interval
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>1</td>
<td>D$_t$-tissue interval$^*$</td>
<td>0.0003</td>
<td>3.10</td>
<td>(1.69, 5.68)</td>
</tr>
<tr>
<td></td>
<td>K-ras 12 mutation</td>
<td>&lt;0.0001</td>
<td>6.49</td>
<td>(2.79, 15.07)</td>
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<tr>
<td></td>
<td>Curative R$_s$</td>
<td>&lt;0.0001</td>
<td>0.10</td>
<td>(0.04, 0.26)</td>
</tr>
<tr>
<td>2</td>
<td>D$_t$-tissue interval$^*$</td>
<td>0.0002</td>
<td>3.25</td>
<td>(1.74, 6.09)</td>
</tr>
<tr>
<td></td>
<td>Any ras mutation</td>
<td>0.0003</td>
<td>3.46</td>
<td>(1.77, 6.73)</td>
</tr>
<tr>
<td></td>
<td>Curative R$_s$</td>
<td>&lt;0.0001</td>
<td>0.13</td>
<td>(0.05, 0.33)</td>
</tr>
<tr>
<td>3</td>
<td>K-ras 12 mutation</td>
<td>0.0003</td>
<td>3.90</td>
<td>(1.85, 8.21)</td>
</tr>
<tr>
<td></td>
<td>Curative R$_s$</td>
<td>&lt;0.0001</td>
<td>0.16</td>
<td>(0.08, 0.32)</td>
</tr>
<tr>
<td>4</td>
<td>Any ras mutation</td>
<td>0.0052</td>
<td>2.29</td>
<td>(1.27, 4.13)</td>
</tr>
<tr>
<td></td>
<td>Curative R$_s$</td>
<td>&lt;0.0001</td>
<td>0.19</td>
<td>(0.10, 0.37)</td>
</tr>
</tbody>
</table>

$^*$ The model parameters ($b_i$) were converted to relative risks by computing $exp(b_i)$ where $exp(x) = 2.7182^x$.

$^*$ The 95% confidence interval for the relative was computed as $[exp(b_{b1}), exp(b_{b2})]$ where $b_{b1} = b_i - 1.96$ [estimated standard error ($b_i$)] and $b_{b2} = b_i + 1.96$ [estimated standard error ($b_i$)].

$^*$ Interval between diagnosis and tissue acquisition.

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3 Our unpublished observation in collaboration with Dr. C. Kadoyama.
shown to be a negative prognostic factor in certain types of human cancers (22–25). While multiple genetic alterations have been described in lung cancers (26), c-myc amplification is a negative prognostic factor for SCLC (24), and ras mutations are a negative prognostic factor for NSCLC.

In conclusion, ras gene mutations are a negative prognostic factor among patients with NSCLC, irrespective of the treatment intent. NSCLC patients with ras mutations, due to their poor prognostic outcome, comprise an important subset that may be suitable for new investigational therapeutic approaches.

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References

ras Gene Mutations in Non-Small Cell Lung Cancers Are Associated with Shortened Survival Irrespective of Treatment Intent

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