Localized Adenocarcinoma of the Lung: Oncogene Expression of erbB-2 and p53 in 150 Patients

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ABSTRACT

Historical information and pathological material from 150 consecutive patients with localized adenocarcinoma of the lung was collected to evaluate oncogene expression of erbB-2 and p53, and erbB-2 gene amplification. Pathological material after resection was reviewed to verify histological staging, and patient follow-up was complete in all cases for at least 68 months. Immunohistochemical expression of erbB-2 (HER-2/neu) and p53 oncogene expression was performed on two separate paraffin tumor blocks for each patient with normal lung as control. Gene amplification of erbB-2 was measured after DNA extraction from 20-μm sections of erbB-2-positive and -negative tumors. All analyses were blinded and included Kaplan-Meier survival estimates with Cox proportional hazards regression modeling. Two adequate blocks of tumor and normal lung were available for 138 (92%) patients. Immunohistochemical identification of expression of p53 was observed in 49 (37%) patients and erbB-2 in 17 (13%) patients. DNA dot blot analyses were performed on 17 erbB-2-positive and 13 randomly selected erbB-2-negative tumors. There was 1 (6%) of 17 erbB-2-positive tumors with 4-fold erbB-2 gene amplification. Actual 5-year survival was 63% and actuarial 10-year survival was 59% for the entire population of 150 patients. Significant univariate predictors (P < 0.05) of cancer death were the presence of symptoms, tumor size >3 cm, poor differentiation, visceral pleural invasion, and p53 expression. Multivariate analysis associated symptoms and p53 expression as independent factors with decreased survival. Thus, this project examined p53 and erbB-2 expression in patients with localized adenocarcinoma and associated p53 status with survival. Multicenter collection of data should allow the development of a model of cancer recurrence in this most common lung cancer.

INTRODUCTION

The most common cause of cancer mortality in women and men in the United States is NSCLC. Since 1970, there has been an increase in the incidence of adenocarcinoma, such that it is now the most common cell type of NSCLC. Although several studies in the medical literature have detailed the impact on survival by the routine pathological features of adenocarcinoma, little data are available which include molecular biological and genetic markers. Additionally, previously reported series of adenocarcinoma of the lung include patients with positive lymph nodes, which is such an important determinant of survival that it may overshadow other factors.

The first purpose of this project was to evaluate the expression of two molecular markers (erbB-2 and p53) and measure gene amplification in those patients with erbB-2-positive tumors. The second purpose was to define factors which impact on survival in a cohort of patients with lymph node-negative adenocarcinoma using these data with routine historical and pathological information. (Stage I was chosen to eliminate the confounding influence of positive lymph nodes or distant metastases.)

PATIENTS AND METHODS

Population

From January 1, 1980 until December 31, 1988, 1928 consecutive patients with documented NSCLC were seen at a single institution. Of these, 537 (27%) were adenocarcinoma of either acinar (n = 454), papillary (n = 11), clear cell (n = 3), or bronchioloalveolar (n = 69) histology. In order to analyze subtypes, only the two most common histologies were included (acinar or bronchioloalveolar; n = 523). Pathological review of these cases revealed 150 patients with negative hilar and mediastinal nodes (T1–2,N0,M0). The mean age was 62 ± 8 (range, 34–81) years, and all patients had complete follow-up data through August 1, 1994 (at least 68 months). Demographics are shown in Table 1. There were 82 males (55%) and 68 females (45%) and a mean tumor size of 2.9 ± 1.8 (range, 1.0–13.0) cm. All patients had an ECOG performance status of 0 or 1 and normal abdominal computed tomograms and radionuclide bone scan. If any mediastinal adenopathy greater than 1 cm was visualized on computed tomography, operative sampling was performed to verify stage I.

Immunohistochemistry

Immunohistochemical analyses were performed on two paraffin blocks of resected lung tissue per patient obtained via an approved Human Subjects Review Committee protocol. Two
different types of genetic markers were chosen for this analysis: proto-oncogene erbB-2 (HER-2/neu) and tumor suppressor gene p53.

### Tissue Preparation

The staining procedure has been described in detail (3–6): paraffin microtome sectioning (4–6 μm) and slide labeling; deparaffinization with xylene and ethanol; antigen retrieval with 5 min of microwave and phosphate-buffered NaCl wash; incubation with the primary mAb or control antibody: p53, mouse mAb (PAb 1801; Oncogene Science, Mineola, NY); erbB-2, rabbit polyclonal antibody to p185neo (PAB; Triton Bio-sciences, Alameda, CA); Incubation with secondary antibody (either goat anti-mouse IgG or goat anti-rabbit IgG); development with Elite Universal kit (Vector Laboratories, Burlingame, CA); Incubation with secondary antibody; and diaminobenzidine-H2O2; and counterstain with methyl green.

### Slide Evaluation

These immunohistochemical methods have been developed and validated in breast and ovarian cancer, and, therefore, these were used as positive controls. Blocks of normal lung from each case served as negative controls. Slides were read immediately by two independent observers and classified as either positive or negative (widespread staining with a subjective intensity of at least + + + on a 0 to + + + scale by both observers). Cases were considered positive if both blocks were graded positive (‡‡‡) or if either block was graded positive (+ + + or ++++) by two independent observers and classified as either positive or negative cases served as negative controls. Slides were read immediately after dehydration with xylene and ethanol; antigen retrieval with microwave and phosphate-buffered NaCl wash; incubation with the primary mAb or control antibody: p53, mouse mAb (PAb 1801; Oncogene Science, Mineola, NY); erbB-2, rabbit polyclonal antibody to p185neo (PAB; Triton Bio-sciences, Alameda, CA); Incubation with secondary antibody (either goat anti-mouse IgG or goat anti-rabbit IgG); development with Elite Universal kit (Vector Laboratories, Burlingame, CA); and diaminobenzidine-H2O2; and counterstain with methyl green.

### Statistical Analysis

Patient data were acquired by retrospective chart review. Follow-up data were obtained by direct patient contact by the Tumor Registry, and no patient was lost to follow-up. Overall cancer-specific survival was defined as from the date of operation to the date of cancer death. An observation was censored at the last follow-up if the patient was alive or the patient had died from a cause other than NSCLC. The Kaplan-Meier product-limit estimator was used to estimate cancer-specific survival curves for subgroups of patients with stage I NSCLC as defined by the following variables: age, gender, smoking history (0/1–20 pack-years/21–50 pack-years/>50 pack-years) and presence of symptoms (ECOG status 0 or 1). Pathological variables included histological subtype (acinar or bronchioloalveolar), tumor size (T1 for tumors ≤3 cm/T2 for tumors >3 cm), differentiation with standard pathological criteria (well/moderate/poor; Ref. 8), visceral pleural invasion, high mitotic rate (15 or more mitotic figures per 10 × 200 microscope fields), and the presence of vascular invasion into the pulmonary arteries or veins. Immunohistochemical variables included presence of immunostaining for erbB-2 and p53. The log rank test was used to compare these subgroups with respect to cancer-specific survival (9, 10). Using Cox’s proportional hazards model and spline transformations of the covariables, the linear and nonlinear effects of age were examined individually (11, 12).

The joint effect of covariables which were significant at the 0.25 level in univariate analysis were examined using stepwise Cox regression. The 0.10 level of significance was used entering or removing a covariable from this model.

### RESULTS

There were two (1.3%) perioperative (30-day) deaths due to myocardial infarctions. No adjuvant therapy was given after resection. Median follow-up was 61 (range, 0.2–169) months. At last follow-up, 53 patients were alive and free of cancer, 41 patients died of other causes without evidence of cancer and 56 (37%) patients had died of cancer. The actual 5-year survival was 63%, while the actuarial 10-year survival was 59% (Fig. 1). Significantly decreased overall survival (P < 0.05) was associated with ECOG status 1 (Fig. 2A), tumor size >3 cm (Fig. 2B), and poor and visceral pleural invasion. The relationship of sections were cut and placed in tubes. The sections were deparaffinized by successive changes in xylene and then digested with 500 μg proteinase K and 0.5% SDS overnight at 50°C. Digested sections were extracted with phenol/chloroform/isoamyl alcohol and then precipitated and washed with ethanol. The integrity of the DNA was checked by agarose gel electrophoresis and quantitated by spectrophotometry.

### Quantitation of erbB-2 Gene Copy Number

The quantitative dot blot analysis has been previously described in detail (6, 7). Briefly, serial 2-fold dilutions of the DNA were spotted onto duplicate nitrocellulose filters. One filter was hybridized with a cDNA encoding the human erbB-2 gene, and the other was hybridized with a human mos gene probe. By comparison of the relative signal intensity between the erbB-2 and mos genes, accurate gene copy numbers were determined. For a positive result, 4-fold or more amplification was required.
several of the variables to survival including male gender, >15 mitoses/microscope field, and vascular invasion approached significance ($P < 0.1$; Table 2).

**Immunohistochemistry.** Immunohistochemical staining for $p53$ and $erbB-2$ were completed in specimens from 138 (92%) patients. Inadequate paraffin blocks were obtained for the remaining 12 patients. Acinar and bronchioloalveolar types expressed $erbB-2$ and $p53$ at the same rates, for an overall incidence of 37% ($n = 49$) and 13% ($n = 17$), respectively. A significantly decreased survival was observed for the patients with $p53$-positive tumors ($P < 0.02$; Fig. 3A). A trend toward a decreased overall survival was observed in the 17 patients who were $erbB$-2 positive (Fig. 3B). No significant covariance was observed between $p53$ and $erbB$-2 immunostaining in this group of adenocarcinomas.  

**Prot-Oncogene DNA Analysis.** One (6%) of 17 (6%) patients who was $erbB$-2 positive was observed to have a 4-fold amplification. No other amplification more than 2-fold was observed in the $erbB$-2-positive group, and no amplification was observed for the 13 $erbB$-2-negative tumors.

**Multivariate Analysis.** Cox proportional hazards regression analysis was used to defined factors which were independent predictors of cancer death. After correction for multiple comparisons, only the presence of symptoms (ECOG status 1) and expression of $p53$ were significant (Table 3).

**DISCUSSION**

The purpose of this project was to define a set of genetic markers in localized adenocarcinoma of the lung and to use these data to more accurately define factors which significantly impact survival. Molecular biological techniques have been utilized extensively in adenocarcinoma of the breast. These techniques are now being applied to adenocarcinoma of the lung, and it would be interesting to compare and contrast results for these two distinct types of adenocarcinoma. Patients were chosen in this study with localized lung cancer (pathological stage I) to eliminate the confounding influence of positive nodes, distant metastases, or poor functional class (ECOG status 2 or greater). In fact, these 150 patients were in a good or excellent shape without any symptoms which limited daily activities.

Squamous cell and small cell were more commonly observed cell types of lung cancer in the United States (1). However, over the last two decades, adenocarcinoma has become the most common cell type. Adenocarcinoma has always been characterized as the most common cell type of NSCLC in women and included the majority of nonsmoking-related lung cancers. However, since 1970 there has been a dramatic increase in the rate of lung cancer in women to the point that it surpassed breast cancer as the number one cancer killer in 1987 (1). It appears that the increase in lung cancer in women and the overall increase in adenocarcinoma may be related, since they occurred simultaneously. Therefore, more data are needed which examine adenocarcinoma of the lung in a modern cohort of patients.

Several large retrospective series of patients with stage I NSCLC are available in the literature. Unfortunately, only a few utilized multivariate analyses and even fewer have examined adenocarcinoma. Pairolero et al. (13) observed an overall survival of 65% at 5 years for 328 stage I NSCLC patients, which included 93 adenocarcinomas. No separate analysis was undertaken for adenocarcinoma, the population included patients with positive hilar lymph nodes, and the only independent predictors of decreased survival were positive nodes and tumor size ($B_T$, $\leq 3$ cm), and $T_2$ ($>3$ cm). Ichinose et al. (14) observed a significant decrease in survival for poor differentiation and aneuploidy using multivariate analysis on 151 total patients, which included 90 adenocarcinomas. No difference in survival was observed for adenocarcinoma or squamous cell carcinoma (14). Sorensen et al. (15) examined 17 variables with multivariate analysis in 137 patients with stage I and II adenocarcinoma of the lung. Only positive
A Kaplan-Meier survival estimates stratified by expression of p53 (A) and the expression of erbB-2 (B). Vertical tick marks, censored observations.

Table 2  Factors for decreased overall cancer-specific survival (5- and 10 years)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Subset</th>
<th>n</th>
<th>Actual 5-yr (%)</th>
<th>Actuarial 10-yr (%)</th>
<th>P</th>
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<tr>
<td>Gender</td>
<td>Female</td>
<td>68</td>
<td>71</td>
<td>68</td>
<td>0.08</td>
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<td></td>
<td>Male</td>
<td>82</td>
<td>57</td>
<td>53</td>
<td></td>
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<tr>
<td>Symptoms</td>
<td>Absent (ECOG 0)</td>
<td>106</td>
<td>77</td>
<td>73</td>
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<tr>
<td></td>
<td>Present (ECOG 1)</td>
<td>44</td>
<td>24</td>
<td>23</td>
<td>0.95</td>
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<tr>
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<td>Acinar</td>
<td>107</td>
<td>61</td>
<td>58</td>
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<tr>
<td></td>
<td>Bronchioloalveolar</td>
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<td>59</td>
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<td>≤3 (T1)</td>
<td>102</td>
<td>68</td>
<td>65</td>
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<tr>
<td></td>
<td>&gt;3 (T2)</td>
<td>48</td>
<td>52</td>
<td>46</td>
<td></td>
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<tr>
<td>Differentiation</td>
<td>Well</td>
<td>42</td>
<td>66</td>
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<tr>
<td></td>
<td>Moderate</td>
<td>64</td>
<td>69</td>
<td>62</td>
<td></td>
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<tr>
<td></td>
<td>Poor</td>
<td>44</td>
<td>51</td>
<td>46</td>
<td>0.006</td>
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<tr>
<td>Pleural invasion</td>
<td>Absent</td>
<td>118</td>
<td>68</td>
<td>64</td>
<td></td>
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<tr>
<td></td>
<td>Present</td>
<td>32</td>
<td>45</td>
<td>40</td>
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<tr>
<td>High mitotic rate</td>
<td>Absent</td>
<td>132</td>
<td>65</td>
<td>62</td>
<td>0.24</td>
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<tr>
<td></td>
<td>Present</td>
<td>18</td>
<td>45</td>
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<tr>
<td>Vascular invasion</td>
<td>Absent</td>
<td>132</td>
<td>66</td>
<td>62</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>18</td>
<td>41</td>
<td>40</td>
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<tr>
<td>erbB-2</td>
<td>Absent</td>
<td>121</td>
<td>63</td>
<td>61</td>
<td>0.29</td>
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<td></td>
<td>Present</td>
<td>17</td>
<td>60</td>
<td>38</td>
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<tr>
<td>p53</td>
<td>Absent</td>
<td>89</td>
<td>70</td>
<td>67</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>49</td>
<td>49</td>
<td>48</td>
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Table 3  Multivariate Cox proportional hazards regression analysis

<table>
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<th>Factor</th>
<th>Risk ratio</th>
<th>P</th>
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<tr>
<td>Symptoms (ECOG 1)</td>
<td>4.53</td>
<td>&lt;0.001</td>
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<tr>
<td>p53 positive</td>
<td>1.81</td>
<td>&lt;0.035</td>
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</table>

nodes, poor performance status, and tumors >3 cm were significant (15). Takise et al. (16) studied 75 patients with adenocarcinoma <2 cm in diameter. Thirty-five patients had positive N1 or N2 lymph nodes and nine had chest wall invasion. Independent predictors of cancer death were positive nodes and parietal pleural involvement (T3; Ref. 16). Macchiarini et al. (17) observed that the presence of vascular invasion and mitotic count (≥13/× 200 microscope field) were independent negative indicators of survival in 95 T1 stage I NSCLC patients. No difference was observed for the 30 patients with adenocarcinoma (17). A multicenter study by the Lung Cancer Study Group, which included more than 5 years of follow-up data on 201 T1 stage I patients, demonstrated no difference in survival for 119 nonsquamous patients compared to 82 patients with squamous cell lung cancer (18, 19).

The analysis of this study population revealed a number of factors that were significantly associated with a decreased survival. These included the presence of symptoms, visceral pleural invasion, poor differentiation, and T2 size. When the cell types of adenocarcinoma were separated, no difference in survival was noted for acinar or bronchioloalveolar types. However, there was a significantly higher percentage of nonsmokers in the bronchioloalveolar population. A retrospective study of 205 patients with bronchioloalveolar carcinoma also suggested a relationship with nonsmokers (20).

Beyond the presentation and routine pathological variables, this project included data on expression of a proto-oncogene, erbB-2, and a tumor suppressor gene, p53. The overexpression of the erbB-2 (HER-2/new) gene, which has significant sequence homology with the gene for the epidermal growth factor receptor (EGFr, erbB-1), was shown by Kern et al. (21) to be a negative prognostic indicator for 10 of 29 patients with advanced adenocarcinoma of the lung (P < 0.04). Since they included all stages of cancer, the possible presence of nodal or distant metastases confounds interpretation of the results. Also, the small patient population (n = 29) makes interpretation of the

References.

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data more difficult. Our series of 150 patients with adenocarcinoma of the lung, included only stage I patients, and we observed a trend toward decreased survival for 17 erbB-2-positive patients. This trend might reach statistical significance with a larger population. Of interest, Yu et al. (22) observed that the metastatic potential of NCI-H460 human NSCLC cells was dramatically increased with transfection with the erbB-2 gene. These laboratory observations support a significant role in tumor invasion and metastasis for lung cancers which overexpress erbB-2.

Earlier work had demonstrated a 2–32-fold amplification of the erbB-2 gene and high expression (+ + +) of erbB-2 protein in 30 of 130 patients with breast cancer. No amplification was observed in an additional 29 cases with moderate expression (+ +) of the erbB-2 protein (7). In the present study, only 1 (6%) of 17 adenocarcinoma specimens graded as positive (+ + or +++) for immunostaining by erbB-2 had a 4-fold erbB-2 gene amplification. Using a similar technique, Shiraiishi et al. (23) found erbB-2 amplification in only 1 of 51 adenocarcinoma specimens examined, verifying the rarity of erbB-2 gene amplification in adenocarcinoma of the lung.

Detectable expression of the p53 protein correlates with point mutations in this tumor suppressor gene because the mutant p53 protein is more stable and resists usual degradation pathways. Nonmutant (wild-type) p53 protein is rapidly degraded and does not reach detectable concentrations. Quilan et al. (24) demonstrated a significant relationship between survival and positive immunostaining for p53 in 49 patients compared to the remaining 65 p53-negative patients. Ebina et al. (25) observed p53 expression in 35% of 71 patients with stages I-II NSCLC, and there was a decreased survival for these patients compared to the rest of the population (P < 0.01). Data from our population of 138 adenocarcinoma patients revealed p53 expression in 49 patients (37%). More important, both univariate and multivariate analyses identified p53 expression as a risk factor for cancer death. The presence of symptoms was the only other variable associated with a decreased survival using multivariate analysis. Also, analysis of covariance did not reveal an association between p53 and erbB-2 expression in these patients with adenocarcinoma of the lung. A similar result was observed for a series of 230 women with stage I or II breast cancer, suggesting that these two genes may act through different mechanisms (26).

Understanding the molecular biology of adenocarcinoma of the lung becomes more important as the rate of this tumor increases with the ultimate goal of developing a reproducible, prospective model of cancer recurrence in localized adenocarcinoma of the lung.

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