Prognostic Value of Nuclear DNA Content and Expression of the ras Oncogene Product in Lung Cancer

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ABSTRACT

We evaluated the prognostic significance of nuclear DNA content by flow cytometry and ras oncogene expression in paraffin-embedded sections of tumors obtained surgically from 112 non-small cell lung cancer patients. Sixty-five (77%) of the 84 tumors had DNA aneuploid patterns that were statistically higher in adenocarcinoma than in squamous cell carcinoma. Of the 91 patients analyzed immunohistochemically using anti-ras M, 21,000 protein (p21) monoclonal antibody rp-35, positive reactions (weak and strong) were observed in 56% of squamous cell carcinomas and 68% of adenocarcinomas. A better 5-yr survival rate was observed in the DNA diploid group (61%) than in the DNA aneuploid group (35%) (P < 0.01). Patients with p21-negative tumors survived significantly longer (5-yr survival rate of 64%) than did those with p21-weak tumors (38%, P < 0.05) or those with p21-strong tumors (12%, P < 0.01). Cox's multivariate analysis showed that DNA ploidy, ras p21 expression, and the stage of the disease were significant prognostic factors for survival. However, the DNA content was not a major independent prognostic factor in adenocarcinoma. The intensity of ras p21 expression was not correlated with nuclear DNA content. These results suggest that DNA content or enhanced ras p21 expression may be different biological markers indicating the malignant potential of lung tumors.

INTRODUCTION

Lung cancer is characterized by a poor prognosis, despite recent improvements in therapeutic procedures. It is, in a sense, a complex of illnesses that has heterogeneous biological functions. This fact complicates the clinical situation and makes diagnosis, treatment, and prevention difficult. In recent years, it has been observed that growth variables of tumors, especially DNA content, are important for the assessment of the prognosis (1–3). Various solid tumors, including lung cancer, with a normal DNA content (DNA diploidy) are associated with a better 5-yr survival rate than those with an abnormal DNA content (DNA aneuploidy) (4–9).

The most frequently identified oncogenes in human solid tumors are members of the ras gene family (10, 11). They encode GTP-binding proteins (p21) and proteins localized at the inner surface of the cell membrane that are thought to be involved in the process of growth-signal transduction across the cell membrane (10). The ras oncogenes acquire their transforming capacity either by a specific point mutation (12) or by enhanced gene expression. The relationship between the abnormality of the ras oncogene and the biological behavior of tumors has recently been investigated in various kinds of human cancers (13–15). With the use of anti-ras p21 MoAbs, the prognostic value of p21 expression has also been assessed in several human cancers (16–19). We previously examined the expression of ras p21 in frozen sections of non-small cell lung cancer immunohistochemically (20, 21), using anti-p21 MoAbs rp-28 and rp-35 (22), and found an association of tumor size or tumor status in the TNM classification with ras p21 expression (21).

In the present study, we evaluated the prognostic significance of the nuclear DNA content, using flow cytometry and ras p21 expression, using MoAb rp-35 to detect p21 in surgically treated patients with lung cancer and further examined whether considering nuclear DNA ploidy and ras p21 expression together can improve the prognostic evaluation of lung cancer.

MATERIALS AND METHODS

Tumors. Tumor specimens from 112 patients (71 male and 41 female patients; average age, 60 yr) with non-small cell lung cancers surgically resected at Hokkaido University Medical Hospital between 1975 and 1985 were analyzed in this study. Tumors were diagnosed histopathologically according to the WHO classification (23) and included 48 squamous cell carcinomas, 56 adenocarcinomas, 5 large cell carcinomas, and 3 adenosquamous cell carcinomas. The postsurgical histopathological TNM staging (pathological stage) were grouped according to the International Union against Cancer classification (24). Most of the patients (who underwent surgery) were treated with combination chemotherapy [5-fluorouracil (10 mg/kg i.v.):cyclophosphamide (4 mg/kg i.v.):mitomycin C (0.04 mg/kg i.v.):chromomycin (0.01 mg/kg i.v.)] for postsurgical treatments for 12 mo.

Flow Cytometry. Suspensions of single nuclei were made from the paraffin blocks according to the method described by Hedley et al. (25), with some modifications. A total of one to five blocks confirmed histopathologically to contain adequate malignant tumor tissue were selected from each tumor. A 30-μm section was cut from each paraffin block. The sections were washed twice for 30 min in xylene to remove the paraffin and then rehydrated by repeated treatment with decreasing concentrations of ethanol (100%, 95%, 80%, 70%, and 50%). Mechanical disaggregation with metal mesh was performed in 50% ethanol, followed by enzymatic dispersion with 0.5% pepsin (pH 1.5) at 37°C for 60 min. After dispersion, the samples were filtered through 40-μm nylon mesh and stained with 50 μg/ml of propidium iodide containing 100 μg/ml of RNase at 4°C for 30 min. The count of cell nuclei was analyzed by a Model 50H Cytofluorograf flow cytometer (Ortho Instruments, Westwood, MA). The excitation wavelength was 488 nm from an argon-ion laser, and the red fluorescence was measured beyond 630 nm through a long-pass filter. An internal standard was provided by the normal diploid pulmonary parenchymal cells present in each sample. The DNA content of each cell subpopulation was expressed as the DNA index, i.e., the ratio between the mode of the aneuploid peak and that of the normal peak in the DNA histogram. DNA diploid tumors were characterized by the presence of one G0-G1 peak, and their DNA indices were equal to 1.00. Tumors were considered to be DNA aneuploid if one or more distinct separate G0-G1 peaks of DNA aneuploid cells were present. For confirmation of the accuracy of DNA measurements, the CV was calculated for the tumor G0-G1 peak in each DNA histogram (26).

Immunohistochemical Procedure. The avidin-biotin-complex immunoperoxidase method (27) was performed on 5-μm sections of formalin-fixed, paraffin-embedded tissue. The primary antibody utilized was rp-
35 mouse IgM monoclonal antibody specific for ras p21, as shown by an enzyme-linked immunosorbent assay and an immunofluorescent assay (22). Desecharifanized and hydrated tissue sections were pretreated with methanol containing 0.3% H2O2 to eliminate endogenous peroxides activity. MoAb rp-35 at a dilution of 1/20 of hybridoma supernatant stock was applied overnight at 4°C after treatment with normal goat serum for 20 min at room temperature. Biotinylated anti-mouse IgM antibody was used as the second antibody, and the slides were then incubated with avidin-biotin-linked peroxides (Vector Labs., Inc., Burlingame, CA). Diaminobenzidine-4 HCl (0.5 mg/ml) with 0.01% H2O2 in 50 mM Tris buffer (pH 7.6) was used as the substrate for detecting localization of the antibody binding. The sections were counterstained with hematoxylin for 10 min. A section of lung cancer cell line A2182, which was strongly reactive with MoAb rp-35, was included in each assay for a positive control. As a negative control, MoAb rp-35 was replaced by an unrelated mouse IgM monoclonal antibody 3.4B2 and/or phosphate-buffered saline. Staining was evaluated by two of us (M. H., H. A.). The intensity of immunohistochemical staining of tumor cells was scored as either negative or equivocal, weak, or strong (Fig. 1). The evaluation of staining intensity was described in detail in our previous papers (20, 21).

Statistical Evaluation. In this prognostic study, only patients with follow-up periods of more than 5 yr after surgery were evaluated. Patients who died within 3 mo after surgery or who died of causes other than lung cancer within 5 yr after surgery also were excluded. Survival was calculated from the day of surgery. The survival curves of the patients were drawn using the Kaplan-Meier method (28), and the statistical evaluation was carried out using the generalized Wilcoxon test (29). Prognostic factors were analyzed using Cox's life-table regression model (stepwise proportional hazards general linear model) and the SAS program package (SAS Institute, Inc., Cary, NC) (30). The P values and significance of data were checked by χ² test.

RESULTS

The results of DNA ploidy and ras p21 staining are shown in Table 1. Of the 84 patients with non-small cell lung cancer analyzed by flow cytometry, 19 (23%) of the tumors were DNA diploid, and 65 (77%) were DNA aneuploid. Of the 19 DNA diploid tumors, only 2 exhibited over 5.1% CV. The CVs of the DNA peaks were within the range of 2.2% to 6.8% (mean, 3.8%). The proportion of DNA aneuploid tumors increased slightly with the advancing stages. However, this trend did not reach a level of statistical significance. Forty patients had squamous cell carcinoma, and 44 patients had adenocarcinoma. The proportion of DNA aneuploid tumors was significantly higher in adenocarcinoma (86%) than in squamous cell carcinoma (68%) (P < 0.05).

Of the 91 patients with non-small cell lung cancer in which ras p21 expression was examined, 33 (36%) showed a negative reaction, 33 (36%) showed a weak reaction, and 25 (28%) showed a strong reaction with MoAb rp-35 (Fig. 1). Positive reactions (weak and strong) were observed in 24 (56%) of 43 squamous cell carcinomas and 27 (68%) of 40 adenocarcinomas. The difference in staining intensity between these two histological types was not statistically significant. The immunoreactivity of MoAb rp-35 was analyzed for all patients in relation to the pathological stage of the disease. Tumors from Stage I patients were less reactive with MoAb rp-35 than those from patients in more advanced stages (I versus II+III+IV, P < 0.05). No significant difference in the correlation was observed between nuclear DNA content and the intensity of ras p21 expression.

Eighty-three patients could be enrolled in the prognostic study of DNA ploidy. The patients were divided into two groups: DNA diploidy (19 cases) and DNA aneuploidy (64 cases). A better 5-yr survival rate was observed in the diploid group (61%) than in the aneuploid group (35%, P = 0.01) (Fig. 2). The relationship between the intensity of ras p21 and the postoperative survival was then analyzed in 94 selected patients. When all histological types were evaluated together, patients with p21-negative tumors had significantly longer survival times than did those with p21-weak tumors (P < 0.05) or those with p21-strong tumors (P < 0.01). The 5-yr survival rates for these three groups were 64%, 38%, and 12%, respectively (Fig. 3).

Analysis of the patients stratified by the resectability of tumors also showed the same results as for those who underwent curative surgery (Table 2). A better 5-yr survival rate was observed in the diploid tumors (70%) than in the aneuploid tumors (48%, P < 0.05). Patients with p21-negative tumors had significantly larger survival time (5-yr survival rate, 74%) than did those with p21-strong tumors (22%, P < 0.05).

When the effects of DNA ploidy, ras p21 staining, stage of disease, tumor status, and node status for all patients were corrected for using Cox's multivariate analysis (Table 3), the pathological stage of the disease (P < 0.0072, χ² = 7.22), DNA ploidy pattern (P < 0.0086, χ² = 6.91), and ras p21 staining (P < 0.0144, χ² = 5.99) remained as significant prognostic factors. For the patients with squamous cell carcinoma, DNA ploidy pattern (P < 0.0009, χ² = 11.03) achieved the highest rank; ras p21 staining (P = 0.0219, χ² = 5.25) and the pathological stage of the disease (P = 0.0317, χ² = 4.61) were the other significant variables, whereas only the pathological stage (P < 0.0135, χ² = 6.10) and ras p21 expression (P = 0.0464, χ² = 3.97) were significant for the patients with adenocarcinoma.

By a combination of DNA ploidy and the intensity of ras p21 expression, the patients were divided into three categories: Group A (10 patients) with tumors having both diploidy and negative or weak p21 expression; Group B (17 patients) having both aneuploidy and negative p21 expression; and Group C (33 patients) having both aneuploidy and weak or strong p21 expression. The Group A patients had the longest survival (5-yr survival rate, 100%). This was followed by the Group B patients (53%) and the Group C patients (21%) (A versus B, P < 0.01; A versus C, P < 0.001) (Fig. 4).

DISCUSSION

Aneuploidy has been reported to occur in 45% to 90% of non-small cell lung cancers (7–9). Our results demonstrated the occurrence of DNA aneuploidy in 96 of 125 (77%) patients with non-small cell lung cancer. These discrepancies are prob-

### Table 1 DNA ploidy and immunoreactivity of MoAb rp-35 in relation to histological type and stage

<table>
<thead>
<tr>
<th>DNA ploidy</th>
<th>ras p21 staining intensity</th>
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</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>Aneuploid</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>13 (33)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>6 (14)</td>
</tr>
<tr>
<td>Stage (pathological)</td>
<td>I</td>
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<tr>
<td>I</td>
<td>9 (32)</td>
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<tr>
<td>II</td>
<td>1 (10)</td>
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<tr>
<td>III</td>
<td>9 (23)</td>
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<tr>
<td>IV</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>19 (23)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, percentage.*

*P < 0.05.
Fig. 1. Immunoperoxidase staining of lung cancer with anti-p21 MoAb rp-35: A, negative staining in squamous cell carcinoma tissue; B, moderate staining in adenocarcinoma tissue; and C, strong staining in squamous cell carcinoma tissue. x 100.

Fig. 2. Survival curves of all patients with non-small cell lung cancer subdivided by DNA ploidy (P < 0.01).

Fig. 3. Survival curves of all patients with non-small cell lung cancer stratified by the staining intensity of MoAb rp-35 (negative versus weak, P < 0.05; negative versus strong, P < 0.01).

Recently, only a few studies (5, 6, 9) have related DNA content to the histopathological differences among primary lung cancers. Sahin et al. (31) reported that DNA ploidy provides an independent prognostic variable for patients with squamous cell carcinoma of the lung, but in non-small cell lung cancer, DNA ploidy was not a significant variable. We also previously reported (32) that DNA content is an independent prognostic factor in squamous cell lung carcinoma, but not in adenocarcinoma of the lung.

We analyzed some important prognostic factors by the Cox proportional hazards linear model which should serve to compensate for the small tumor panel. In the present study, DNA ploidy and ras p21 expression were shown to be independent prognostic variables and the major determinants of survival for all patients by the multiple regression model along with the pathological stage of the disease, which is generally accepted as a major prognostic factor for survival in non-small cell lung cancer. However, the proportion of DNA aneuploid tumors was significantly higher in adenocarcinoma (86%) than in squamous cell carcinoma (68%). In the patients with squamous cell carcinoma, furthermore, the pathological stage, DNA ploidy pattern, and ras p21 staining were important factors. For patients with adenocarcinoma, only the pathological stage and ras p21 staining were significant, but the DNA content was not a major prognostic factor for survival. These results indicate that the clinical value of the DNA content is different in squamous cell carcinoma and adenocarcinoma.

Recent immunohistochemical or immunoblotting analyses utilizing anti-ras p21 monoclonal antibodies have related p21 expression to tumor characteristics. Enhanced p21 expression had a correlation with more malignant tumor phenotypes, such as deeper tumor invasion and advanced Dukes' stage in colorectal cancer (16), and larger tumor size and lymph node involvement in breast cancer (17). A few studies (33–35) have demonstrated enhanced p21 expression in lung cancer, but its
clinical significance remains to be defined.

The present immunohistochemical study demonstrated that ras p21 expression was associated with the stage of the disease, and survival analysis demonstrated that higher levels of p21 expression correlated with shorter survival time for patients with non-small cell lung carcinoma. The difference between ras p21 expression in squamous cell carcinoma and adenocarcinoma was not statistically significant. On multivariate analysis for survival, ras p21 staining was a significant prognostic factor both for patients with squamous cell carcinoma and for those with adenocarcinoma. Therefore, ras p21 expression is considered to be an important and stage-independent indicator of prognosis in lung cancer.

Recent biological studies have revealed that ras oncogene expression correlates with metastatic potential as well as tumorigenicity in certain experimental systems (36). It has also been shown that ras oncogenes increase the resistance of recipient cells to ionizing radiation (37) and to chemotherapeutic agents (38). These biological properties of ras oncogenes may affect the clinical status, response to treatment, and the outcome of patients whose tumors have ras oncogene abnormality. The intensity of ras p21 expression was not correlated with nuclear DNA content. By a combination of DNA ploidy and the intensity of ras expression, however, the patients with tumors having both diploidy and negative or weak p21 expression had the longest survival (5-yr survival rate, 100%). These results suggest that DNA content and enhanced ras p21 expression may be different biological markers indicating the malignant potential of lung tumors.

In conclusion, the measurement of DNA content, immunohistochemical analysis using MoAb rp-35, and the stage of the disease considered together may improve prognostic evaluation of lung cancer.

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