Expression of Nucleolar Protein p120 in Human Lung Cancer: Difference in Histological Types as a Marker for Proliferation

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INTRODUCTION

Pleomorphic and hyperactive nucleoli are major cytological characteristics of tumor cells (1). This increased proliferation-associated nucleolar activity relates, in part, to increased ribosomal biogenesis, the major function of the nucleolus. In the course of development of antinucleolar antibodies, a human specific proliferation-associated nucleolar protein, p120, has been identified (2). This protein was detected in most tumor cells but not in quiescent cells or normal resting cells (2). The expression of p120 is regulated during cell cycle, with a dramatic increase at the G1-S boundary (3-5). Overexpression of p120 protein transformed NIH3T3 cells and resulted in colony formation in soft agarose and rapidly growing tumors in nude mice (6). Antisense constructs of p120 cDNA and p120 antisense oligodeoxynucleotide inhibited the growth of human cancer cells in vitro and in vivo (7, 8). The p120 expression was associated with cell proliferation rate in human breast cancer cell lines in vitro (9). A retrospective study of human breast cancer also indicated that the p120 expression may be a prognostic marker of breast cancer patients (10).

Although p120 was extensively studied in human breast cancer in vitro and in vivo, only a limited study has been performed in other types of human cancer (11, 12). In this study, a high percentage of p120-positive cells was observed in squamous cell carcinoma in contrast to lower percentages in adenocarcinoma and large cell carcinoma in resected human lung cancer tissues. Furthermore, in vitro study demonstrated that the p120 expression was strongly correlated with the cell proliferation rate of human lung cancer cell lines, although these are different histological types.

MATERIALS AND METHODS

Immunohistochemical Staining of p120 in Surgically Resected Lung Cancer Specimens. Cryostat specimens were obtained from 37 patients with lung cancer, who underwent surgical treatment in the Hospital of Institute of Development, Aging, and Cancer, Tohoku University, and affiliated hospitals from September 1993 to December 1994. The thirty-seven lung cancer specimens consist of 19 adenocarcinomas, 13 squamous cell carcinomas, and 5 large cell carcinomas. Tissues were fixed with 2% formaldehyde and permeabilized with acetone. After removing endogenous peroxidase with 0.3% hydrogen peroxide in methanol, the slides were incubated with 10% normal rabbit serum for blocking nonspecific reactions. The slides were then incubated with mouse antihuman p120 monoclonal antibody (p120 MAb2; Oncogene Science, Cambridge, MA) overnight at 4°C and incubated with biotin-conjugated goat anti-mouse (IgG + IgA + IgM) antibody (Nichirei, Tokyo, Japan). Blotting

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2 The abbreviation used is: MAb, monoclonal antibody.
was developed with Histofine SAB PO(M) kit (Nichirei). The tissues were finally counterstained with Mayer-hematoxylin solution. Two examiners independently counted p120-positive cells of more than 500 cancer cells. Labeling index of p120 was calculated as the percentage of p120-positive cells in the total number of cancer cells.

**Cell Culture and Determination of Doubling Time.** Small cell lung carcinoma cell lines (SBC-3 and Lu65), lung adenocarcinoma cell lines (A549 and RERF-LCMS), a squamous cell lung carcinoma cell line (Sq-19), and a normal lung fibroblast cell line (MRC-5) were obtained from Japanese Cancer Research Resources Bank. A squamous cell lung carcinoma cell line (HS-24) was kindly provided by Dr. Muley (Heidelberg, Germany). Small cell lung carcinomas and squamous cell lung carcinomas were cultured in RPMI 1640 with 10% fetal bovine serum; lung adenocarcinomas and a normal lung fibroblast were cultured in Eagle's Minimum Essential medium with 10% fetal bovine serum. Doubling time of each cell line was determined, based on cell growth curves.

**Northern Blot of p120 mRNA.** Total RNA (20 μg) isolated with Isogen (Wako, Tokyo, Japan), was denatured, fractionated in a 1.2% agarose gel containing formaldehyde, and transferred to a nylon membrane. The p120 cDNA fragment (0.5 kb) from pBS p120 was used for the probe (13). The p120 cDNA fragment was labeled with [α-32P]dCTP by random primer labeling kit (Takara, Tokyo, Japan) and hybridized in Quick hyb buffer (Stratagene, La Jolla, CA) at 68°C for 2 h. The membrane was rehybridized with β-actin cDNA probe (1.4 kb) as internal control. Radioactivities were determined with Bio Imaging Analyzer BAS-2000 (Fuji, Tokyo, Japan).

**Western Blot of p120 Protein.** Cells were solubilized in Laemml buffer at 95°C for 5 min (14). The samples were loaded onto 7.5% polyacrylamide/SDS gel, electrophoresed, and transferred to a nylon membrane. After incubation with p120 MAb at room temperature for 2 h, the blot was probed by Phosphatase Substrate System (Kinkegaard & Perry Laboratory, Gaithersburg, MD). The density of each band was measured by a densitometer for quantification. A duplicated gel was stained with Coomassie Blue to assess the equal protein loading.

**Cell Cycle Analysis.** Cell suspension (1 × 10^5) of each cell line was fixed with 50% ethanol, washed with PBS solution, and treated with 0.2 ml of RNase solution in PBS (1 mg/ml) at 37°C for 30 min. Cellular DNA was stained with propidium iodide solution (50 mg/ml) at 4°C for 2 h. Cells were analyzed with FACSsort (Nippon Becton Dickinson, Tokyo, Japan), and cell cycle fractions were determined.

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**RESULTS**

**Immunohistochemical Staining of Nucleolar Protein p120.** Nucleoli of cancer cells were positively stained with p120 MAb in all cancer tissues examined (Fig. 1, A and B). In normal lung tissues, only alveolar macrophages were weakly stained (Fig. 1C). Expression levels of the p120 protein in lung cancer tissues were quantitated by counting p120-positive cells in cancer tissues. When the labeling index of p120 was compared to histological types, the index of squamous cell carcinoma was significantly higher than that of adenocarcinoma or large cell carcinoma (67.7 ± 12.4% versus 35.3 ± 12.6%, 30.1 ± 17.3%, respectively; \( P < 0.01 \); Fig. 2). Intensity of p120 staining of nucleoli in squamous cell carcinoma was stronger than the intensity in adenocarcinoma or large cell carcinoma (Fig. 1, A and B). No other clinicopathological factors, such as Tumor-Node-Metastasis status, stage, and differentiation, were correlated with the labeling index of p120.

When seven lung cell lines cultured in vitro were stained with p120 MAb, the nucleoli of all cell lines were positively stained (data not shown). The labeling index of p120 was over 80% in all cell lines examined. There was no significant difference in p120 labeling index among seven cell lines.

*Fig. 1* Immunohistochemical staining of nucleolar protein p120 in human lung cancer tissues and normal lung tissue. Tumor sections were fixed and stained with p120 MAb as described in “Materials and Methods.” A, tumor tissue of squamous cell carcinoma; B, tumor tissue of adenocarcinoma; C, normal lung tissue.
Fig. 2 Scattergram showing percentage of p120-positive cancer cells in three different histological types of lung cancer. Each circle indicates the percentage of p120-positive cancer cells in a tumor tissue. The mean values showed a significant difference between squamous cell carcinoma and adenocarcinoma or large cell carcinoma.

Fig. 3 Expression of p120 mRNA in human lung cell lines. Total RNAs (20 μg) were electrophoresed, transferred to a nylon membrane, and hybridized with α-32P-labeled p120 cDNA probe (upper panel). The membrane was rehybridized with β-actin probe as internal control (lower panel). Lane 1, Lu65; Lane 2, SBC-3; Lane 3, HS-24; Lane 4, Sq-19; Lane 5, A549; Lane 6, RERF-LCMS; Lane 7, MRC-5.

Table 1 Quantitative analyses of p120 mRNA and p120 protein in human lung cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>p120 mRNA</th>
<th>p120 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lu65 (Sm)</td>
<td>12.7</td>
<td>4.26</td>
</tr>
<tr>
<td>SBC-3 (Sm)</td>
<td>29.0</td>
<td>5.53</td>
</tr>
<tr>
<td>HS-24 (Sq)</td>
<td>11.3</td>
<td>5.16</td>
</tr>
<tr>
<td>Sq-19 (Sq)</td>
<td>4.21</td>
<td>1.95</td>
</tr>
<tr>
<td>A549 (Ad)</td>
<td>7.14</td>
<td>5.26</td>
</tr>
<tr>
<td>RERF-LCMS (Ad)</td>
<td>2.50</td>
<td>3.42</td>
</tr>
<tr>
<td>MRC-5</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

a The respective levels of p120 mRNA and p120 protein in MRC-5 cells were considered as 1.00. The values for other cell lines were given as a ratio to that of MRC-5 cells. Data represent the mean of duplicate experiments.

Sm, small cell carcinoma; Sq, squamous cell carcinoma; Ad, adenocarcinoma.

Fig. 4 Expression of p120 protein in human lung cell lines. Cell extracts (1 × 10^6) were loaded onto a 7.5% polyacrylamide/SDS gel, electrophoresed, and transferred to a nylon membrane. The blot was developed with p120 MAb as described in "Materials and Methods." M, protein marker; Lane 1, Lu65; Lane 2, SBC-3; Lane 3, HS-24; Lane 4, Sq-19; Lane 5, A549; Lane 6, RERF-LCMS; Lane 7, MRC-5.

Fig. 5 Analyses of p120 Expression by Northern Blot and Western Blot in Lung Cell Lines. Although all cell lines examined expressed p120 mRNA transcripts, expression levels depended on each cell line (Fig. 3). When relative amounts of p120 mRNA transcripts were determined as a ratio to MRC-5 cells, a small cell carcinoma cell line SBC-3 showed the highest level of p120 mRNA transcripts, whereas an adenocarcinoma cell line RERF-LCMS and normal lung fibroblast MRC-5 showed fewer p120 mRNA transcripts (Table 1). The p120 protein was detected as a single band in all cell lines as shown in Fig. 4. Quantitative analysis of p120 protein expression by densitometry revealed that a small cell carcinoma cell line SBC-3 expressed the highest level of p120 protein, whereas a squamous cell carcinoma cell line Sq-19 and normal lung fibroblast MRC-5 expressed lower levels (Table 1). The MRC-5 cell line expressed lowest p120 in both mRNA and protein levels. The level of p120 mRNA transcripts and level of p120 protein had a positive correlation (r = 0.699), suggesting that p120 is regulated transcriptionally.

Doubling Times and Cell Cycles in Lung Cell Lines. To study a correlation between p120 expression and proliferation rate in human lung cell lines, doubling times and cell cycles were analyzed. Two small cell lung cancer cell lines (SBC-3 and Lu65) and A549 cells grew faster, and their doubling times were shorter than 20 h, whereas Sq-19 and MRC-5 grew slower, and their doubling times were over 30 h (Table 2). The fastest growing SBC-3 cells contained largest S-phase fraction (37.6%), whereas slower growing cells (RERF-LCMS and MRC-5) contained less than 11% S-phase fractions (10.1 and 9.5%, respectively; Table 2). The proportion of S-phase was 25–30% in the other three cell lines (Lu65, HS-24, and A549).

Correlation between p120 Expression and Proliferation Rate. According to the calculated Pearson's correlation coefficient, there was a significant and positive correlation between p120 mRNA expression and the S-phase fraction (r = 0.851, \( P < 0.02 \); Fig. 5; Tables 1 and 2). In addition, p120 protein
expression was significantly and positively correlated with S-phase fraction ($r = 0.869$, $P < 0.01$) or significantly and reversibly correlated with doubling time ($r = -0.928$, $P < 0.01$; Tables 1 and 2). Although the correlation between p120 mRNA expression and doubling time ($r = -0.739$) was also observed, this was not significant ($P = 0.06$).

**DISCUSSION**

Although nucleolar protein p120 was identified in the course of screening of monoclonal antibodies against human nucleoli (1, 2), the physiological function of p120 protein remained unclear. On the basis of sequence homology, Koonin reported that p120 is a methyltransferase, putatively serving as methylase for rRNA (15). Recently, a nucleolar protein in yeast (NOP2) with significant amino acid homology (67%) to human p120 was isolated (16). Although NOP2 was markedly up-regulated during the onset of growth, its overproduction produces no discernible growth phenotype but influences the morphology of the nucleolus. Lung cancers are unique in that different histological types are derived from different carcinogenic mechanisms at different anatomical distributions, and that tumor biology against clinical intervention is characteristic of the group of certain histological types. However, only limited markers are available to assess these biological characteristics, other than morphological interpretation.

In the present study, using 37 frozen specimens from surgically resected lung cancer, immunohistochemical evaluation of p120 labeling indices in the nucleoli of cancer cells revealed a significant difference among different histological types: squamous cell carcinoma (67.7%), adenocarcinoma (35.5%), and large cell carcinoma (30.1%). This is the first report that demonstrates the variation in the expression level of p120 among different histological types of lung cancer. Only a few studies of p120 expression were performed in human cancer tissues (10, 11). A study using operated human breast cancer tissues indicated that p120 was a prognostic marker in patients with breast cancer (10). When p120 expression was examined in benign hyperplastic prostate sections and prostate carcinoma sections, p120 was negative or very weakly positive in benign hyperplastic prostate, whereas p120 was positive in prostate carcinoma (11). They also reported an increase in labeling index in proportion to cancer grading. Because no other clinicopathological factors were correlated with p120 labeling indices in this study, the reason why p120 labeling index in squamous cell carcinoma is high remains open for further investigation.

Recently, Costa et al. (17) evaluated cell proliferation using surgical specimens of NSCLC and reported that the S-phase cell fraction was not related to clinical stage but was significantly associated with tumor histology. A lower median [3H]thymidine labeling index value (3.2%) was observed in adenocarcinomas than that (13.6%) in squamous cell carcinomas, although the ranges partly overlapped. In addition, Usuda et al. (18) reported that the tumor doubling time in squamous cell carcinoma (80.3 days) was significantly shorter than that in adenocarcinoma (163.3 days). In this context, high labeling index of p120 in squamous cell carcinoma in this study may reflect higher proliferation rates.

Our *in vitro* study demonstrated that the protein level of p120 was directly correlated with the proliferation rate and S-phase fraction of human lung cancer cells. The level of p120 mRNA was also positively correlated with S-phase fraction. Fonagy et al. (9) reported similar results where p120 expression was highly correlated with proliferation capacity in six human breast cancer cell lines. The role of p120 in cell proliferation was also demonstrated in previous studies (5–8). These data support the thesis that p120 is necessary for cell cycle progression and may be a proliferation marker in both normal and tumor cells.

In conclusion, nucleolar protein p120 is expressed highly in the specimens of squamous cell carcinoma compared to adenocarcinoma or large cell carcinoma. The p120 expression is positively correlated with the growth capacity of human lung cancer cell lines *in vitro*, and p120 has an important function in proliferation of tumor cells.

**Table 2** Doubling times and cell cycle fractions

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Doubling time (h)</th>
<th>G0-G1 (%)</th>
<th>S (%)</th>
<th>G2-M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lu65 (Sm'</td>
<td>19.8 ± 2.0</td>
<td>35.7</td>
<td>27.9</td>
<td>36.4</td>
</tr>
<tr>
<td>SBC-3 (Sm)</td>
<td>15.5 ± 2.1</td>
<td>40.2</td>
<td>37.6</td>
<td>22.2</td>
</tr>
<tr>
<td>HS-24 (Sq)</td>
<td>26.5 ± 3.5</td>
<td>50.6</td>
<td>28.5</td>
<td>20.8</td>
</tr>
<tr>
<td>Squ-19 (Sq)</td>
<td>33.6 ± 3.4</td>
<td>67.7</td>
<td>18.5</td>
<td>13.8</td>
</tr>
<tr>
<td>A549 (Ad)</td>
<td>19.8 ± 2.0</td>
<td>62.7</td>
<td>25.8</td>
<td>11.5</td>
</tr>
<tr>
<td>RERF-LCMS (Ad)</td>
<td>27.3 ± 1.3</td>
<td>60.7</td>
<td>9.5</td>
<td>29.8</td>
</tr>
<tr>
<td>MRC-5</td>
<td>44.0 ± 3.0</td>
<td>76.1</td>
<td>10.1</td>
<td>13.8</td>
</tr>
</tbody>
</table>

a Data represent the mean ± SD of triplicate experiments.

b Data represent the mean of duplicate experiments.

Sm, small cell carcinoma; Sq, squamous cell carcinoma; Ad, adenocarcinoma.
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