Altered Retinoblastoma Protein Expression and Proliferative Activity in Urethane Induced Mouse Lung Tumorigenesis

Jin-Haeng Chung, M.D.¹, Ja-June Jang, M.D.², Min Jae Lee, Ph.D.² and Eui Keun Ham, M.D.²

¹Department of Pathology, Korea Cancer Center Hospital, Seoul; ²Department of Pathology, Seoul National University College of Medicine, Seoul, Korea

**Purpose:** Lung cancer develops through a multistage process involving the accumulation of diverse genetic alterations. To gain an understanding of the roles played by tumor suppressor gene proteins and proliferating cell nuclear antigen (PCNA) in chemical carcinogen-induced mouse lung tumorigenesis, we examined the expression of retinoblastoma protein (Rb), p53, and PCNA in normal lung tissues and urethane-induced mouse lung tumors.

**Materials and Methods:** ICR mice were given urethane by intra-peritoneal injection, and sacrificed at 5, 13, 21, 31, and 37 weeks following treatment. Sequential morphological changes and the immunohistochemical expression of Rb protein, p53, and (PCNA), during mouse lung tumorigenesis, were examined.

**Results:** During the carcinogenesis, sequential histological changes from hyperplasia of type II pneumocytes, to adenomas, and ultimately to overt adenocarcinomas were noted. Intense nuclear staining of the Rb protein was observed in normal and hyperplastic alveolar epithelial cells and adenomas. In adenocarcinomas, the Rb protein expression was significantly diminished. The p53 mutant protein was not detected in any lesion. The PCNA labeling index increased along with the advance in the histological grade.

**Conclusion:** The above results indicate that mouse pulmonary adenocarcinomas develop through premalignant lesions, and down-regulation of the Rb protein expression may be implicated in the urethane-induced mouse lung tumorigenesis. In addition, the PCNA labeling index may reflect the malignant potential during the tumor progression. *(Cancer Research and Treatment 2002;34:258-263)*

**Key Words:** Lung neoplasm, Tumorigenesis, Urethane, Rb protein

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**INTRODUCTION**

The administration of the alkylating carcinogen, urethane (ethyl carbamate) induces lung tumors in susceptible rodent strains (1). The tumors exhibit morphological, histogenic and biochemical features similar to those seen in human pulmonary adenocarcinomas. Moreover, a number of genetic alterations associated with human lung cancer have been found in pre-malignant lesions and adenocarcinomas in the lungs of animals (2,3). Therefore, the murine model of pulmonary carcinogenesis provides a useful experimental system for studying the molecular and morphological changes associated with human pulmonary adenocarcinomas (4).

During multistage carcinogenesis, the activation of proto-oncogenes and the inactivation of tumor suppressor genes are important steps towards the more malignant tumor stage (3,5). The p53 and retinoblastoma (Rb) genes are the most extensively studied tumor suppressor genes (6). Gene alterations, such as missense point mutations or deletion, are known to result in uncontrolled cell proliferation, which contribute to malignant transformation (5). However, comparatively controversial results concerning the p53 or Rb gene alterations have been reported for chemically induced lung tumors in mice (2,3). No mutations in the p53 tumor suppressor gene have been found in chemically induced mouse lung tumors (7,8), with the exception of urethane-induced tumors at a late stage of development (3). Loss of heterozygosity at the Rb locus exists in hybrid mouse lung adenocarcinomas (9). The Rb mRNA levels in mouse lung tumors are reduced compared to those in the normal lung, but Rb protein levels are similar in normal mouse lung to in lung tumors (2).

Information on the cell kinetics may provide further understanding of the biological behavior of tumors. Many studies have reported the relationship between the malignant potential of neoplasms, and cell proliferative kinetics (10). Proliferating cell nuclear antigen (PCNA) is an auxiliary protein for the DNA polymerase delta, which plays an important role in DNA...
synthesis, and is thought to be synthesized in nuclei, particularly during the proliferative period of the late G1- and S-phases (11,12). PCNA has been used to estimate the malignant potential of some neoplasms (13,14). To gain an understanding of the roles played by tumor suppressor gene proteins, and PCNA, in chemical carcinogen-induced mouse lung tumorigenesis, we examined the expression of the Rb, p53, and PCNA in normal lung tissues and urethane-induced mouse lung tumors. In addition, we examined the correlation between the PCNA labeling index and the respective lesions in tumorigenesis.

**MATERIALS AND METHODS**

1) **Induction of urethane-induced lung tumors**

Three-week-old male ICR mice were purchased (Clea Japan, Inc., Tokyo, Japan), housed in a controlled environment maintained at 25±2°C with 30~60% relative humidity and an alternating 12 h light-dark cycle. After a 1 week acclimation period, the mice were treated with urethane (Sigma Co., St. Louis, MO), dissolved in saline, by intraperitoneal injection at a dose of 0.5 mg/gm, twice a week for 4 weeks. As a control group, male ICR mice of the same age, untreated with urethane, were used. Groups of mice, urethane-treated and controls, were sacrificed by carbon dioxide inhalation at 5, 13, 21, 31, and 37 weeks post-treatment (Table 1). The lungs were removed, tumors were excised, and the lung tissues and tumors were divided into two portions. One portion was frozen immediately in liquid nitrogen and stored at -80°C. The remaining portion of each tissue was fixed in 10% neutral buffered formalin for 24 h, then dehydrated and embedded in paraffin. Hematoxylin and eosin-stained sections were examined microscopically to confirm the histology of each type of tissue. The tumors were classified by cytological and histological criteria according to Foley et al. (15).

2) **Immunohistochemical techniques**

Five-micrometer paraffin-embedded tissue sections were deparaffinized in xylene and hydrated in a graded ethanol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Tissue sections were heated in 10 mM sodium citrate, pH 6.0, in a microwave oven for 10 min to expose the antigens. Sections were then washed with Tris-buffered saline (TBS). The streptavidin-biotin-peroxidase complex technique (universal LSAB kit, DAKO, Carpinteria, CA) was used for immunohistochemical stain. The sections were incubated overnight at 4°C with the primary antibodies; anti-Rb (C-15, 1 : 100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p53 (CM1, 1 : 100, Novocasta, Newcastle, UK), and anti-PCNA (PC 10, 1 : 100, DAKO). Sections were then washed with and incubated with biotinylated secondary antibody for 30 minutes at room temperature. After washing, the sections were incubated with peroxidase-labelled streptavidin at room temperature for 30 minutes. Diaminobenzidine hydrogen peroxidase was used as a chromogen and sections were counterstained with hematoxylin. For the PCNA and Rb, normal bronchiolar epithelium was used as a positive internal control. For the p53, sections of rat hepatocellular carcinoma, which had been shown to be p53-positive, were used as a positive control. The primary antibodies were omitted in each immunohistochemical stain as a negative control.

3) **Rb immunohistochemical staining analysis**

The methods we used to stain the Rb nuclear protein, in paraffin-embedded tissue sections, have been described previously (16). In all cases, adequate nuclear staining was obtained in adjacent normal lung tissues, which represented an internal positive control for the Rb protein staining in each section. A lesion was considered to be Rb-positive (Rb+), if the Rb protein staining was heterogeneous, with a portion of the cells showing typical Rb nuclear protein staining. Lesions were considered as Rb-negative (Rb-) only if all cells in the lesion showed no Rb nuclear protein staining and if the adjacent normal cells were positively stained with Rb protein.

4) **Scoring of p53 immunoreactivity**

When the nuclei of cells were stained diffusely brown, regardless of the staining intensity, the cells were considered to be positive for the accumulation of the p53 mutant protein.

5) **Determination of PCNA labeling index**

Brownish nuclear staining, regardless of staining intensity, was considered to be the evidence of PCNA expression. The PCNA labeling index was determined by scanning areas with uniformly stained cells at a low magnification, followed by counting of the cells at ×400. Nuclei with brown staining were scored as positive, and the PCNA labeling index was calculated as the percentage of positive nuclei in a total of 500 cells. In small hyperplastic lesions, however, the cells in the entire lesion had to be counted to determine the PCNA labeling index. The number of cells counted in each of these hyperplastic lesions ranged from 200 to 500.

6) **Statistical analyses**

One-way ANOVA and Chi-square tests were used to determine the statistical significance of the groups. A p value less than 0.05, by a two-tailed test, were considered significant.

**RESULTS**

1) **Induction of lung tumors by urethane**

In urethane-treated mice, grossly visible mass formation was noted after 13 weeks post-treatment. At 13 weeks following the urethane-treatment, multiple small nodules had developed macroscopically in 6 out of 10 mice. After 21 weeks, following
Fig. 1. Gross morphology of lung tumor in ICR mice treated with urethane. (A) Multiple small nodules at 21 weeks after treatment. (B) Large masses at 31 weeks after treatment.

Fig. 2. Sequential change in urethane-induced lung lesions. (A) Alveolar epithelial hyperplasia. Note that the cells are plump and cuboidal (H&E, ×200). (B) Adenoma. Contiguous alveolar spaces are obliterated by proliferating epithelial cells (H&E, ×40). (C) Adenocarcinoma. Infiltration into the adjacent alveolar space is seen (H&E, ×40).
the administration of urethane, grossly detectable multiple masses were found in all the mice, and the masses increased in size with time (Fig. 1). Microscopic examination revealed alveolar hyperplasias in 10 mice (total 12 mice), at 5 weeks following the administration of urethane. Proliferation of swollen type II pneumocytes in hyperplasias were seen along the intact alveolar septa (Fig. 2A). The cells lining the alveolar walls were relatively uniform and cuboidal without cytologic atypia or nuclear pleomorphism. The margins of the hyperplasias were ill defined and irregular. Multiple small nodules found at 13 weeks post-treatment, were diagnosed as adenomas (Fig. 2B). Cells comprising of these nodules (adenoma) were generally well differentiated, but occasionally exhibited a mild degree of cytologic atypia. Compression of the adjacent pulmonary parenchyma was frequently observed. Overt adenocarcinomas appeared at 31 and 37 weeks post urethane-treatment in 19 mice. In the remaining mice, multiple adenomas with multifocal alveolar hyperplasias had developed. The adenocarcinomas comprised of cells with various degrees of cytologic atypia and heterogeneous growth patterns. Nucleoli were distinct, and large bizarre and pleomorphic nuclei were often noted. Scattered mitotic figures were also observed, with an increase in the nuclear/cytoplasmic ratio. Invasion into the surrounding lung parenchyma or bronchioles was occasionally present (Fig. 2C). There were no concomitant metastatic foci or pathological lesions in any other organs. We selected 39 hyperplasias, 56 adenomas, and 19 adenocarcinomas for immunohistochemical examination of the Rb protein, p53 protein, and PCNA.

2) Immunohistochemical detection of Rb protein

Intense nuclear staining of the Rb protein was observed in lung tissue from the controls and the urethane-treated mice. Nuclear staining was observed in the alveolar epithelial, bronchial epithelial and endothelial cells. The hyperplasias and adenomas showed positive immunostaining for the Rb protein, but the intensity of the staining of the adenomas were heterogeneous, and slightly decreased, compared to the epithelial cells of normal lung tissues. The adenocarcinomas showed significantly diminished immunostaining in multifocal areas, with ten (53%) being negative against Rb immunostaining in tumor cells. While every single cell in these lesions showed negative nuclear Rb protein staining, the adjacent normal cells, serving as positive internal controls, expressed the Rb protein (Fig. 3).

3) p53 nuclear accumulation

None of the hyperplastic, adenomatous or adenocarcinomatous lesions were positive for the p53 protein. In the rat hepatocellular carcinoma, used as positive control, many tumor cells were positive for the nuclear p53 staining.

4) PCNA labeling index

Table 2 shows the PCNA labeling indices of the lung lesions. A few PCNA-positive cells were scattered in normal bronchiole and alveoli. In the normal lung parenchyma of the control mice, the PCNA labeling indices ranged from 6.4% to 19.5% (average, 12.7%). The number of positive cells increased in accordance with the more histologically advanced grades, and the proliferating cells, with large nuclei, were encountered more often, and were strongly positive for the PCNA (Fig. 4). The mean ± standard deviation (SD) values for the PCNA labeling indices in hyperplasias, atypical adenomatous hyperplasias, and adenocarcinomas were 35.2 ± 16.9% (n=39), 48.5 ± 18.9% (n= 56) and 76.3 ± 16.3% (n=19), respectively. The ranges of the PCNA labeling indices were 9% to 80%, 6% to 84%, and 36% to 92%, in hyperplasias, atypical adenomatous hyperplasias and adenocarcinomas, respectively. Although the ranges apparently overlap, the PCNA labeling indices were significantly different
Table 2. PCNA labeling index in lesions during mouse lung tumorigenesis

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Hyperplasia (n=39)</th>
<th>Adenoma (n=56)</th>
<th>Adenocarcinoma (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA labeling index</td>
<td>35.2±16.9</td>
<td>48.5±18.9</td>
<td>76.3±16.3</td>
</tr>
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*Labeled nuclei per 500 cells (mean±SD)
† Significantly greater than the hyperplasia group, p < 0.05.
‡ Significantly greater than the other groups, p < 0.001.

Fig. 4. Immunohistochemical staining for PCNA in adenocarcinoma. Tumor cells with large nuclei tend to be more strongly positive (>200).

between the hyperplasias and adenomas (p < 0.05), and between the adenomas and adenocarcinomas (p < 0.001). The index was also significantly different between normal epithelia and hyperplasias (p < 0.05). However, there was no difference between Rb+ and Rb- adenocarcinomas in the PCNA labeling index.

DISCUSSION

To study the pathogenesis and histogenesis of neoplastic development, it is essential to investigate the biological properties of the early neoplastic lesion. Like other types of cancer, lung cancer develops through a multistage process involving the accumulation of diverse genetic alterations that affect several proto-oncogenes and tumor suppressor genes (17,18). Although there has been a significant increase in pulmonary adenocarcinomas (19), the scarcity of preneoplastic materials in these tumors make it difficult to examine molecular and genetic alterations systematically. It has been well documented that chemical carcinogen-induced lung tumors in mice arise as hyperplasias, progress to adenomas, and ultimately result in adenocarcinomas (15). Although human pulmonary adenomas have not been considered as premalignant lesions, mouse lung tumor models have been used for studying the molecular and morphological changes associated with human pulmonary adenocarcinomas (2,4). In the present study, we examined the Rb and p53 protein expressions, and the PCNA labeling index, in a urethane-induced mouse lung tumorigenesis model. Alteration to the Rb gene has been documented in various types of cancers, including lung cancer (16,18,20,21). However, only a few studies have suggested that the inactivation of the Rb might be implicated in mouse lung carcinogenesis (2,9,22). For example, the Rb mRNA expression was reduced 6- to 10-fold, compared to that of normal lung tissue, in AC3, BALB/c and A/J mice lung adenomas (3). We found that the Rb protein expression was decreased in adenocarcinomas, which corresponds with previous studies (2,9,22). In this study, however, there was no altered expression of the Rb protein in adenomas, with the exception of a slightly weakened staining intensity. The reasons for this difference are not clear. Possible explanations include the different mouse strain, the different time points for sacrificing animals or the different methods used for the detection of the Rb inactivation (immunohistochemistry vs. DNA or RNA analyses). Since individual tumors contain cells of varying degrees of malignancy, and reductions or absences of gene expressions measured in whole tumor tissues may be compromised by subpopulations of cells that exhibit varying degrees of expression. We think, immunohistochemical techniques would be more appropriate for examining the Rb protein. Our results indicate the Rb inactivation may occur relatively late during mouse lung tumorigenesis, and the decreased expression of the Rb protein may be implicated in the urethane-induced mouse lung tumorigenesis.

Alterations of the p53 gene have been shown to be one of the most common molecular biological changes in human lung cancer (23,24). In contrast to human lung cancer, the frequency of p53 mutations, in chemically induced murine lung cancers, have not been well documented (3,7,8). In this study, there was no detectable mutant p53 protein in any of the lesions. As we did not search for p53 mutation at the DNA level, the presence of silent mutation of the p53 can not be ruled out. Whereas Horio et al. (3) reported p53 mutations in urethane-induced mouse lung tumors, other studies have failed to detect p53 mutations in chemically induced mouse lung tumors (7,8). Further studies are required to examine the incidences of p53 mutations during mouse lung tumorigenesis.

PCNA is a 36 kDa acidic non-histone nuclear proteins which functions in virtually all phases, but is particularly strongly expressed in the late G1- through S-phases of the cell cycle (11,12). It is frequently used as a marker of cell proliferation and is known to correlate well with the histological grade of malignancy in humans (13,14) and mouse lung tissue (25). In this study, we observed higher levels of the PCNA labeling index in adenocarcinomas than in adenomas or hyperplasias. The PCNA labeling index was also higher in adenomas than in hyperplasias. The PCNA expression increased progressively from normal lung tissue, through to preneoplastic lesions, and finally to adenocarcinomas. These results suggest that high
PCNA labeling indices may be useful indicator of a high proliferative activity and a more advanced histological grade.

CONCLUSION

During mouse lung carcinogenesis, sequential morphological changes, from hyperplasias, to adenomas, and to adenocarcinomas, were noted with the expression of the Rb protein being reduced in adenocarcinomas. This finding suggests down-regulation of the Rb protein might contribute to the tumor progression in this animal model. In addition, a high PCNA labeling index was found to correlate well with a more histologically advanced grade, and therefore, it may reflect a malignant potential.

REFERENCES