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Analysis of $p21^{Waf1/Cip1}$ Expression in Normal, Premalignant, and Malignant Cells during the Development of Human Lung Adenocarcinoma

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Our studies suggested that adenocarcinoma of the peripheral lung mostly develops by several steps from atypical adenomatous hyperplasia through early adenocarcinoma to overt adenocarcinoma, and that some p53 abnormalities play an important role in this progression. In the present study, we examined by immunohistochemistry the expression of p53-inducible cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ (p21) in the cells at various developmental stages of lung adenocarcinoma (32 lesions of adenomatous hyperplasia, 14 of early adenocarcinoma, 23 of well differentiated adenocarcinoma, and 17 of moderately or poorly differentiated adenocarcinoma) in comparison with 19 reactive proliferative lesions and analyzed the relationship between p53 and p21 expression. Bronchioalveolar cells in the normal lung expressed very little or no p21 and no p53 expression. In not only reactive but also neoplastic lesions regardless of their developmental stage, the cells expressed p21 at various frequencies. The average labeling indices ranged from 5.4 to 13.8%, and there was no significant difference between any of these categories. The expression of p21, however, tended to be relatively low in moderately and poorly differentiated adenocarcinomas (5.5%) compared to well differentiated adenocarcinomas (12.2%), and high-level p21 expressors (10% ≤ positive cells) were more frequent in the latter group (1 of 17 (6%) *versus* 8 of 23 (35%); $P < 0.05$), suggesting that p21 expression is affected by the degree of differentiation of the neoplastic cells. Although the correlation was positive between the expression of p21 and p53 in reactive lesions ($r = 0.88$; $P < 0.001$), none was found in neoplastic lesions at any step or grade ($-0.12 \leq r \leq 0.26$). These results indicated that p21 expression depends upon p53 expression in reactive lung cells, whereas p21 expression is at least in part independent of that of p53 from the earliest to the most fully developed step of lung adenocarcinoma tumorigenesis. We concluded that disruption of the p53-dependent cell cycle regulation is a very early event in the tumorigenesis of lung adenocarcinoma. (Am J Pathol 1997, 151:461–470)

Mutations and deletions of the p53 gene are the most common genetic alterations hitherto documented in a wide range of human malignancies, including lung cancers.[1–7] Wild type p53 is activated by DNA damage, and either induces cell replication arrest, allowing DNA repair to occur, or facilitates apoptosis in cells with irreversibly damaged DNA.[8–11] A loss of normal p53 function leads to the survival and reproduction of cells harboring genetic errors, resulting in an increased probability that variant cell populations with a growth advantage will be propagated.[8,9,12] This mechanism plays an important role in neoplastic development and progression. The *Waf1/Cip1* gene is transcriptionally activated through p53 by DNA damage, and the product p21 inhibits the activity of cyclin D-Cdk4/6 and cyclin E-Cdk2 complexes, inducing arrest of the cell cycle at the G_1 and G_1/S phases, respectively.[13–17] Thus, p21 functions as a critical downstream effector in the p53-specific pathway of growth control in mammalian cells. Accumulated evidence shows that p21 can also be induced in p53-independent pathways by growth factors and agents inducing differentiation and growth arrest.[18–25] In some tissues and cells, p21 induction is associated with terminal differentiation[18,25–27] and senescence.[28]

Although the histogenesis of lung adenocarcinoma has long been controversial, various studies have suggested that adenocarcinomas of the peripheral lung, like many other cancers, mostly develop by multiple steps from atypical adenomatous hyperplasia (AAH) through early (*in situ*) carcinoma to overt carcinoma.[29,30] We revealed that some p53 gene abnormalities play an important role in this progression.[29,30] Thus, it is necessary to

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investigate p21 expression in relation to p53 status at different developmental steps of lung adenocarcinoma to determine whether the p53-dependent pathway of cell cycle regulation is intact and whether it is perturbed, and at which step(s) this occurs. Presently this has been examined only in a few neoplasms. The expression of p21 is reportedly dependent upon normal p53 function in colorectal carcinomas[26] but is independent in pancreatic carcinomas,[19] gastric neoplasms,[31] and non-small cell lung carcinomas.[22]

In the present study, we investigated by immunohistochemistry the expression of p21 in a variety of proliferative lesions of the peripheral lung, including reactive proliferation, AAH, and early and overt adenocarcinomas, and compared the results with those of p53 expression. Loss of heterozygosity (LOH) in the p53 gene was also analyzed in selected lung lesions and polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) was used to detect mutations in p53 gene in some of the lesions. Moreover, p21 expression and proliferative activity was correlated by Ki-67 immunoreactivity.

Materials and Methods

Patients and Tissues

We studied 32 lesions of AAH (17 low-grade AAH and 15 high-grade AAH), 14 of early (*in situ*) adenocarcinoma, 23 of well differentiated adenocarcinoma, 10 of moderately differentiated adenocarcinoma, and seven of poorly differentiated adenocarcinoma (for definitions of each category, see Refs. 29 and 30). These were obtained from 53 patients who underwent lobectomy or pneumonectomy. The patients consisted of 22 men and 31 women whose ages ranged from 28 to 83 years with a mean ± SD of 63.1 ± 11.4 years. Nineteen lesions of reactive bronchioalveolar cell proliferation (six lambertosis, nine localized acute phase interstitial pneumonia without significant fibrosis, and four localized interstitial pneumonia with various degrees of organization) were also studied for comparison. These were obtained from nine patients who underwent surgery for concurrent lung cancer. The patients consisted of five men and four women whose ages ranged from 46 to 69 years with a mean ± SD of 60.4 ± 7.8 years. All samples were collected from the surgical pathology files at the School of Medicine of Yokohama City University, Kanagawa Cancer Center, Kanagawa Cardiovascular and Respiratory Center, and Yokohama Municipal Citizen's Hospital (Yokohama, Japan) between 1988 and 1996. None of the patients had undergone chemotherapy or radiotherapy preoperatively.

The resected lung lobes were fixed by the intrabronchial instillation of 20% neutral formalin, and the tissues were embedded in paraffin. Sections (4 μm thick) were cut and stained with hematoxylin and eosin and examined immunohistochemically. In some cases, fresh tissues were frozen in liquid nitrogen and stored at −80°C until used.

Immunohistochemistry

Primary antibodies used for p53 immunostaining were a mouse monoclonal anti-p53 protein (DO7; Dako A/S, Glostrup, Denmark) at a 1:100 dilution and a rabbit polyclonal anti-p53 protein antibody (CM1; Novocastra Laboratories Ltd., Newcastle, UK) at a 1:500 dilution. These two antibodies are reactive with wild-type and mutant forms of p53 protein. For p21 immunostaining, a mouse monoclonal anti-p21 protein antibody (NCL-WAF1; Novocastra Laboratories Ltd.) was applied at a 1:80 dilution. For detection of Ki-67 antigen, a mouse monoclonal antibody, MIB1 (Immunotech, Marseilles, France), was diluted 1:100.

Paraffin sections were immunostained as described.[29,30] Briefly, sections were deparaffinized with xylene and rehydrated with graded ethanol concentrations. Antigen was retrieved by incubation in 10 mmol/L citrate buffer, pH 6.0, at 95°C for 10 minutes in a microwave oven. After blocking with preimmune goat or rabbit serum, the sections were incubated for 60 minutes at room temperature with the primary antibodies DO7, CM1, and MIB1 or overnight at 4°C with NCL-WAF1. Sections were subsequently stained with a biotinylated secondary antibody and a streptavidin-biotin-peroxidase detection kit (Histofine, Nichirei, Tokyo, Japan), in which diaminobenzidine was the chromogen.

Positive controls included colonic carcinoma tissue for p53 and Ki-67 and normal appendix tissue for p21. Negative control sections were incubated with normal mouse or rabbit serum instead of primary antibodies.

Cells were considered positive when the nucleus was distinctly stained. The labeling index was determined for each of the antibodies by counting 2000 cells in the most representative areas. In smaller lesions, all the cells in the entire sample were counted (300–1000 cells per lesion).

In selected lesions (four in each category), serial sections were cut and stained alternatively for p53 (with DO7) and p21 to evaluate whether these two proteins were coexpressed. In this study, antigen sites were visualized with True Blue (Kirkegaard and Perry Laboratories, Gaithersburg, MD) according to the manufacturer's instructions.

DNA Extraction

For restriction fragment length polymorphism (RFLP) analysis, two to five sections (5 μm thick) were cut from paraffin-embedded tissues and mounted on glass slides. The sections were deparaffinized with xylene, washed with absolute ethanol, stained briefly with toluidine blue, and microdissected. Under the inverted microscope, desired portions of the sections were dissected out with a 20-gauge needle. The samples were suspended in digestion buffer (10 mmol/L Tris-HCl, pH 8.3; 1 mmol/L EDTA; 1% SDS; and 2 μg/μl proteinase K) and incubated at 48°C for 24 to 36 hours. DNA was extracted by the phenol-chloroform method. For small lesions (<1 cm), dissected samples were suspended in the digestion buffer without SDS. After incubation, proteinase K was inactivated by heating at 95°C for 10 minutes, and then

Table 1. Labeling Index for p53 (CM1, DO7), p21, and Ki-67

Histology	*n*	CM1	DO7	p21	Ki-67
Neoplastic	86	9.3 ± 20.2	9.3 ± 18.1	9.8 ± 18.1	7.2 ± 10.7
Low-grade AAH	17	0.5 ± 1.0	1.7 ± 2.9	13.8 ± 24.5	0.6 ± 0.7
High-grade AAH	15	3.1 ± 7.8	0.5 ± 0.7	10.6 ± 18.4	1.3 ± 1.2
E-AC	14	2.1 ± 3.7	5.5 ± 14.2	5.4 ± 8.3	4.4 ± 4.1
W-AC	23	18.7 ± 29.5	17.8 ± 23.3	12.2 ± 18.5	9.1 ± 7.3
M-AC and P-AC	17	17.1 ± 24.0	16.2 ± 22.6	5.5 ± 16.4	18.7 ± 16.9
Reactive	19	1.4 ± 2.4	1.3 ± 1.9	5.7 ± 9.4	7.8 ± 12.5
LIP, acute phase	9	2.7 ± 3.1	2.4 ± 2.2	11.5 ± 11.2	12.1 ± 16.0
LIP, organizing phase	4	0.2 ± 0.4	0.5 ± 0.7	1.2 ± 2.0	9.5 ± 8.9
Lambertosis	6	0.1 ± 0.2	0.0 ± 0.0	0.2 ± 0.3	0.2 ± 0.4

Values are presented as means ± SD (%).
Abbreviations: E-AC, early adenocarcinoma; W-AC, well differentiated adenocarcinoma; M-AC, moderately differentiated adenocarcinoma; P-AC, poorly differentiated adenocarcinoma; LIP, localized interstitial pneumonia.

the samples were centrifuged and the supernatant was used for PCR. DNA was also extracted from normal lung tissues or normal lymph node tissues in the same manner and used for the control. For PCR-SSCP analysis, DNA was extracted from frozen tissues by the phenol-chloroform method.

PCR and RFLP Analysis

Ten lesions of AAH, 10 of early adenocarcinoma, and 23 of overt adenocarcinoma were examined for an LOH in exon 4 of the p53 gene by RFLP analysis. The procedures were performed as described.[32,33] Exon 4 of the p53 gene was amplified with the primer pair 1) 5′-GCTGTCCCCGGACGATATTG-3′ and 2) 5′-AATGCAAGAAGCCCAGACGG-3′ by 40 PCR cycles. Samples were initially denatured before amplification for 5 minutes at 94°C. The cycle conditions were 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The PCR products were digested with *Bst*UI (New England Biolabs, Beverly, MA) overnight at 60°C. The digest was resolved by electrophoresis through a 1% agarose gel in 1× TAE buffer (40 mmol/L Tris; 40 mmol/L acetic acid; and 1 mmol/L EDTA, pH 8.0). The gel was stained with 0.5 μg/ml ethidium bromide and was visualized with an ultraviolet transilluminator.

Polymerase Chain Reaction-Single Strand Conformation Polymorphism

One lesion of high-grade AAH, two of early adenocarcinoma, two of well differentiated adenocarcinoma, three of moderately differentiated adenocarcinoma, and two of poorly differentiated adenocarcinoma were examined for mutations in exon 5 to 8 by PCR-SSCP. DNA samples were amplified in the same way as described above, and [α^{35}-S]dATP and the following primers were used: 1) 5′-TTCCTCTTCCTGCAGTACTC-3′ and 2) 5′-GCCCCAGCTGCTCACCATCG-3′ for exon 5; 1) 5′-CACTGATTGCTCTTAGGTCTG-3′ and 2) 5′-AGTTGCAAACCAGACCTCAG-3′ for exon 6; 1) 5′-GAGGCAAGCAGAGGCTGG-3′ and 2) 5′-CCAAGGCGCACTGGCCTC-3′ for exon 7; 1) 5′-CCTATCCTGAGTAGTGGTAATC-3′ and 2) 5′-GTCCTGCTTGCTTACCTCGC-3′ for exon 8. Ten microliters of labeled PCR products were mixed with 5 μl of stop solution (95% formamide, 0.05% bromophenol blue, 0.05% xylenecyanol, and 20 mmol/L EDTA), boiled for 5 minutes, and cooled on ice rapidly. Samples were run at 16 W on 6% acrylamide gel and autoradiography was performed against X-ray film.

Statistical Analysis

The difference in mean values was analyzed by the Mann-Whitney test. The difference in frequency was analyzed by the Fisher exact test. The Spearman's correlation coefficient was used to determine correlation among the p21, p53, and Ki-67 labeling index values.

Results

Immunohistochemistry and Labeling Index Values

Cells in the normal lung tissue were not stained for p53. In general, cells in AAH and early adenocarcinoma showed absent, rare, or only sporadic nuclear staining for p53, whereas the staining was variable in well, moderately, and poorly differentiated adenocarcinomas ranging from absent or rare to diffuse and, on occasion, dense accumulation of stained cells (clonal pattern). Among the reactive lesions, positive cells were absent or rare in lambertosis and organizing phase pneumonia, but were more frequent, although sporadic, in acute phase pneumonia. The results were mostly identical for the two antibodies, CM1 and DO7, although there was some discrepancy. The p53 labeling indices are summarized in Table 1. Statistically, the combined groups of well, moderately, and poorly differentiated (overt) adenocarcinomas had significantly higher p53 labeling index values than the combined group of AAH and early adenocarcinoma, as well as having p53 labeling index values higher than reactive lesions ($P < 0.05$ each).

Cells in the normal lung tissue showed no staining or very rare nuclear staining for p21 except for the alveolar macrophages and bronchiolar epithelial cells, which

Table 2. Correlation among Labeling Index Values

Histology	CM1 and DO7	CM1 and p21	DO7 and p21	p21 and Ki-67
Neoplastic	0.63*	−0.06	0.08	−0.08
Low-grade AAH	0.35	0.15	0.26	0.36
High-grade AAH	−0.22	−0.09	0.15	0.10
E-AC	0.33	−0.59	0.17	0.16
W-AC	0.61*	−0.13	0.05	0.33
M-AC and P-AC	0.98*	0.00	−0.12	−0.40
Reactive	0.60**	0.64*	0.88*	0.40

*, $P < 0.001$; **, $P < 0.01$ (Spearman's correlation coefficient).
See Table 1 for abbreviations.

showed occasional nuclear staining. The staining for p21 in AAH, early adenocarcinomas, and overt adenocarcinomas was variable ranging from sporadic to diffuse, but positive cells rarely accumulated. No difference was seen in the labeling index for p21 among histological types except for the lower frequencies in early adenocarcinoma and the combined group of moderately and poorly differentiated adenocarcinomas than in the remainder (Table 1). In reactive lesions there was no significant difference in the labeling index for p21 compared with neoplastic lesions. The labeling index in acute phase pneumonia was higher than that in organizing pneumonia and lambertosis.

Nuclei of cells in the normal lung parenchyma were only rarely stained for Ki-67. Combined well, moderately, and poorly differentiated adenocarcinomas showed significantly higher Ki-67 labeling index values than combined groups of AAH and early adenocarcinomas ($P < 0.001$) and reactive lesions ($P < 0.05$) (Table 1). Combined moderately and poorly differentiated adenocarcinomas had significantly higher labeling index values than did well differentiated adenocarcinomas ($P < 0.05$). Early adenocarcinomas showed labeling index values significantly higher than low-grade AAH ($P < 0.01$). Among the reactive lesions, Ki-67 labeling appeared to parallel both the p53 and p21 positivity, except organizing phase pneumonia showed Ki-67 labeling index values comparable to those in acute phase pneumonia.

Relationship between p53 (CM1 and DO7) and p21 Expression

The correlation between CM1- and DO7-labeling index values in reactive lesions and overt adenocarcinomas was positive, especially in combined groups of moderately and poorly differentiated adenocarcinomas (Table 2). There was no correlation between p53 and p21 expression in any category of neoplastic lesions (Table 2 and Figure 1). In contrast, their expression was positively correlated in reactive lesions ($r = 0.64$, $P < 0.001$ with CM1 and $r = 0.88$, $P < 0.001$ with DO7) and the labeling index value for p21 was generally almost equal to or higher than that for p53 being approximately four times as high on average (Table 1). The relationship between p53 and p21 expression was close in reactive lesions at the cellular level according to a topographical analysis of serial sections (Figure 2, a and b). However, their expression was not necessarily associated in the neoplastic lesions (Figure 2, c–h). In both the reactive and neoplastic lesions, some cells expressed p21 separately. Variable numbers of cells positive only for p53 were observed in many but not all neoplastic lesions, whereas such cells were very rare in the reactive lesions.

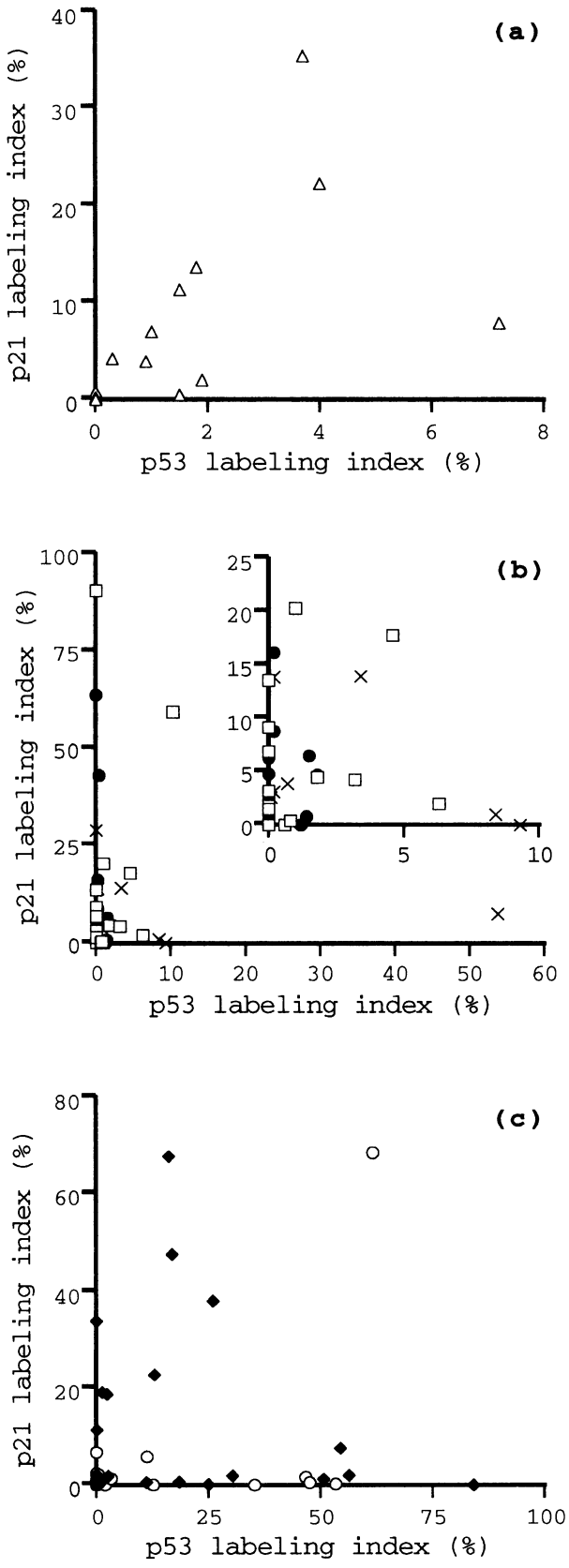
According to the criteria proposed by Doglioni et al,[26] lesions with 10% or more p21 positive cells are considered to express high levels of p21. Lesions with a p53 labeling index above 10% were considered high-level expressors of p53 because all of the labeling index values for p53 in the reactive lesions examined here were below 10%. There was no difference in p21 expression among the high- and low-level p53 expressors (Table 3). However, most of overt adenocarcinomas showing high-level p53 expression (14 of 19) expressed p21 only at a low level. In combined moderately and poorly differentiated adenocarcinomas, only 1 of 17 (6%) lesions expressed high levels of p21, whereas in well differentiated adenocarcinomas, 8 of 33 (35%) lesions expressed high levels of p21. The difference was statistically significant ($P < 0.05$).

RFLP Analysis

Normal lung or lymph node tissues were heterozygous for the polymorphic region in exon 4 of the p53 gene in 11 of 20 (55%) lesions of AAH or early adenocarcinoma and 16 of 23 (70%) lesions of overt adenocarcinoma examined. LOH in exon 4 of the p53 gene was detected in 1 of 7 (9%) AAH lesions (Table 4). This was a low-grade AAH. None of the 4 early adenocarcinomas showed LOH. Among the 11 overt adenocarcinomas, LOH was detected in 5 lesions (31%). All of these 5 lesions were well differentiated adenocarcinoma. None of the 6 moderately differentiated adenocarcinomas showed LOH. In the lesions without LOH, the correlation between p53 and p21 and between Ki-67 and p21 expression was inverse ($P < 0.01$, each) (Table 5).

Polymerase Chain Reaction-Single Strand Conformation Polymorphism

Abnormal band mobilities were not detected by PCR-SSCP in any lesions examined of high-grade AAH, early adenocarcinoma, well differentiated adenocarcinoma, or poorly differentiated adenocarcinoma. Abnormal bands were detected in two of the three lesions (67%) of mod-



erately differentiated adenocarcinoma (one lesion in exon 5 and the other in exon 8) (Table 4).

Discussion

We demonstrated that the overexpression of p53 protein increased significantly from AAH to early (*in situ*) adenocarcinoma and to overt adenocarcinoma.[29,30] It is generally accepted that p53 overexpression reflects missense mutations of the gene[34] or binding of the wild type p53 to viral or cellular inhibitory proteins[35] and thus is associated with a loss of normal function. The results of the present study confirmed our previous findings.[29,30] Furthermore, the results of this study on LOH in the p53 gene were consistent with those of our immunohistochemical studies, although the number of lesions examined was limited. We also investigated mutations in exons 5 to 8 of the p53 gene by PCR-SSCP analysis and found that none of three lesions of AAH or early adenocarcinoma had any, whereas two of seven overt adenocarcinomas did.

The $p21^{Waf1/Cip1}$ is a crucial downstream effector of p53-dependent cell cycle regulation. Only a few investigators, however, have examined the expression of p21 in relation to that of p53 in the respiratory tract epithelium.[22,36,37] With an intact p53-dependent pathway, p21 is expressed in response to activated p53. Hence, p21 expression correlates with that of p53.[36,37] However, when this pathway is disrupted, p53 and p21 expression are not correlated.[22]

As reported,[22,36–38] the results of the present study showed that p21 expression was rare and sporadic in normal bronchioalveolar epithelial cells. As the vast majority of terminally differentiated, ciliated, and type 1 alveolar cells were negative for p21, its expression is unlikely to be associated with terminal differentiation in lung cells. This situation contrasts to that in the colonic epithelium in which p21 expression is important in the induction and maintenance of terminal differentiation.[26,39] The airway epithelium, which is a conditional renewal system and normally shows very low proliferative activity differs remarkably in cytokinetics and perhaps also in its regulatory mechanism from colonic epithelium in which new cells are constantly and continuously produced and the progeny cells differentiate terminally and are lost.[40] In normal lung cells, factors other than p21 may play an important role in regulation of the cell cycle and the maintenance of terminal differentiation.

In reactive lung cells, p21 expression appeared to be dependent upon that of p53. Topographical analyses of serial sections as a rule confirmed an intimate association between p53 and p21 expression at the cellular level, although the p21 labeling index was on average approximately four times as high as that of p53. This discrep-

Figure 1. Relationship between p53 (stained with DO7) and p21 labeling index values. **a:** reactive lesions (△); **b:** low-grade AAH (□), high-grade AAH (●), and early adenocarcinomas (×); **c:** well differentiated adenocarcinomas (◆) and combined moderately and poorly differentiated adenocarcinomas (○). In **b**, the part with p53 labeling index below 10% and p21 labeling index below 25% is also shown at a higher magnification.

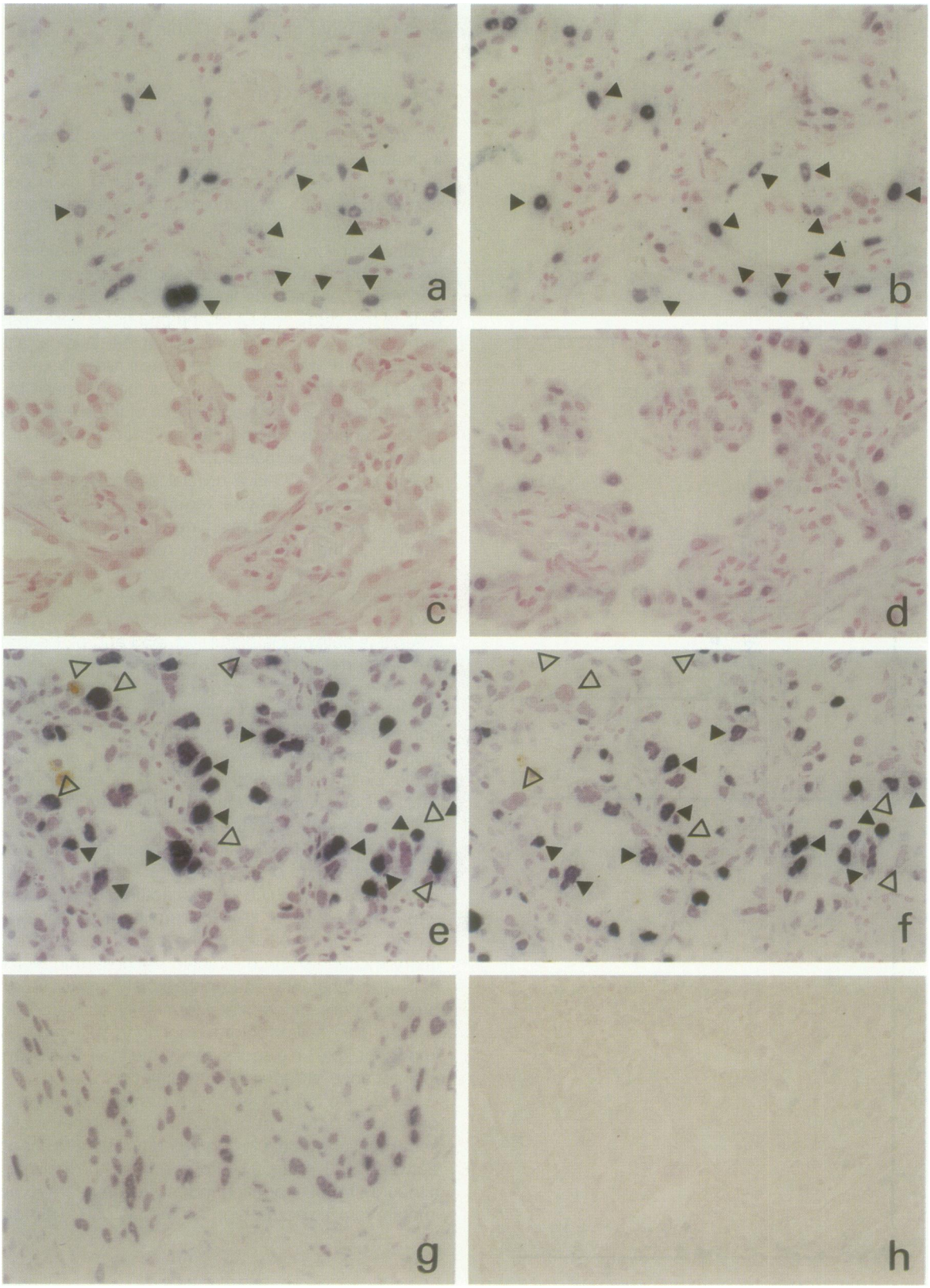


Figure 2. Comparison of p53 and p21 expression at the cellular level in serial sections by immunostaining. p53 (**a, c, e,** and **g**) and p21 immunostaining (**b, d, f,** and **h**). **a** and **b:** Acute phase interstitial pneumonia. p53 (**a**) and p21 (**b**) are coexpressed in many epithelial cells (**solid arrowheads**), whereas some cells express p21 separately (**upper left corner**). **c** and **d:** Atypical adenomatous hyperplasia. Whereas no p53-positive cells are evident (**c**), p21 is expressed in many cells (**d**). **e** and **f:** Well differentiated adenocarcinoma. Cells positive for p53 (**e**) or p21 (**f**) are abundant, p53 and p21 are coexpressed in some cells (**solid arrowheads**), but other cells are positive for either p53 or p21 separately (**open arrowheads**). **g** and **h**: Poorly differentiated adenocarcinoma. Most of cells are intensely positive for p53 (**g**), whereas no p21 immunoreactivity is observed (**h**). Magnification, **a–d** ×280; **e–f** ×250; **g–h** ×280.

Table 3. Frequency Distribution of Lesions According to the Expression Levels of p53 (with DO7) and p21

Histology	*n*	p21 < 10%	p21 ≥ 10%
Neoplastic			
p53 < 10%	65	51 (78%)	14 (22%)
p53 ≥ 10%	21	15 (71%)	6 (29%)
Low-grade AAH			
p53 < 10%	16	12 (75%)	4 (25%)
p53 ≥ 10%	1	0 (0%)	1 (100%)
High-grade AAH			
p53 < 10%	15	12 (80%)	3 (20%)
p53 ≥ 10%	0		
E-AC			
p53 < 10%	13	10 (77%)	3 (23%)
p53 ≥ 10%	1	1 (100%)	0 (0%)
W-AC			
p53 < 10%	11	7 (64%)	4 (36%)
p53 ≥ 10%	12	8 (67%)	4 (33%)
M-AC and P-AC			
p53 < 10%	10	10 (100%)	0 (0%)
p53 ≥ 10%	7	6 (86%)	1 (14%)
Reactive			
p53 < 10%	19	15 (79%)	4 (21%)
p53 ≥ 10%	0		

See Table 1 for abbreviations.

ancy in the absolute labeling index values may have reflected the differences in the amounts of these proteins expressed or may have been caused by the differences in the sensitivity of the detection technique used here. The expression of p53 and p21 was more pronounced in the acute phase than in the organizing phase reactive lesions. Guinee et al[36] and Kuwano et al[37] demonstrated the association of p53 and p21 expression in diffuse alveolar damage and idiopathic pulmonary fibrosis, respectively, and the importance of their expression in induction of apoptosis. These results and our results suggest that in lung cells, DNA is damaged during inflammation, for example by free radicals, and as a result G_1 arrest is induced through a p53-dependent pathway. The concomitant high Ki-67 labeling and high-level p21 expression in some of our reactive lesions do not contradict this interpretation, as the Ki-67 antigen is expressed in the cells at all phases of the cell cycle except G_0.[41]

In premalignant and malignant lung cells, the expression of p21 varied considerably from lesion to lesion. The level of p21 expression did not differ significantly according to the developmental step and the grade of lung

Table 4. LOH and Mutation (Exons 5–8) in p53 Gene and p53, p21, and Ki-67 Expression

Lesion	Grade	CM1	DO7	p21	Ki-67	LOH	Mutation
1	AAH, low-grade	0.0	4.6	17.8	1.7	+	ND
2	AAH, low-grade	3.1	3.2	4.3	0.5	–	ND
3	AAH,high-grade	28.8	0.0	2.4	3.9	–	ND
4	AAH,high-grade	1.8	0.0	4.8	2.0	–	ND
5	AAH,high-grade	0.0	0.2	8.8	2.6	–	ND
6	AAH,high-grade	0.2	0.0	0.5	2.8	–	ND
7	AAH,high-grade	0.0	0.0	0.9	0.0	–	–
8	E-AC	0.0	0.0	28.9	0.2	–	ND
9	E-AC	9.5	53.8	7.5	2.8	–	ND
10	E-AC	1.8	0.1	2.6	0.2	–	ND
11	E-AC	0.1	0.0	0.4	12.4	–	ND
12	E-AC	0.3	8.4	1.0	6.6	NI	–
13	E-AC	0.0	0.0	0.8	0.0	ND	–
14	W-AC	62.2	50.7	1.2	12.4	+	ND
15	W-AC	18.8	16.2	67.8	6.7	+	ND
16	W-AC	45.9	56.4	2.0	21.9	+	ND
17	W-AC	0.2	1.1	0.6	5.7	+	ND
18	W-AC	2.7	16.9	47.6	14.0	+	ND
19	W-AC	57.5	25.0	0.1	3.7	–	ND
20	W-AC	40.5	11.2	0.5	4.9	–	ND
21	W-AC	6.5	2.6	1.8	3.3	–	–
22	W-AC	0.0	0.0	1.2	2.0	–	ND
23	W-AC	0.0	0.1	11.5	1.1	–	ND
24	W-AC	0.8	0.0	2.1	0.9	NI	–
25	M-AC	0.0	0.0	2.2	11.5	–	ND
26	M-AC	8.4	35.3	0.0	17.2	–	ND
27	M-AC	20.5	12.7	0.0	16.8	–	ND
28	M-AC	2.5	1.9	0.0	15.0	–	ND
29	M-AC	0.0	0.0	2.4	1.1	–	–
30	M-AC	1.3	1.0	0.6	7.1	–	+*
31	M-AC	2.0	3.3	1.3	28.0	NI	+†
32	P-AC	74.6	61.7	68.6	0.3	ND	–
33	P-AC	0.4	0.1	1.4	2.4	ND	–
LOH(+) (*n* = 6)		21.6 ± 26.6‡	24.3 ± 23.6	22.8 ± 28.5	10.4 ± 7.2		
LOH(−) (*n* = 21)		8.7 ± 15.6	7.0 ± 14.2	3.9 ± 6.5	5.3 ± 5.7		
LOH(+) overt AC (*n* = 5)		26.0 ± 27.2	28.3 ± 24.0	23.8 ± 31.7	12.1 ± 6.5		
LOH(−) overt AC (*n* = 11)		12.5 ± 19.4	8.2 ± 12.0	1.8 ± 3.3	7.6 ± 6.4		

*, exon 5.
†, exon 8.
‡Values are means ± SD.
Abbreviations: ND, not done; NI, not informative. Others are as shown in the footnotes to Table 1.

Table 5. Correlation among p53 (CM1 and DO7), p21, and Ki-67 Labeling Index Values with Regard to LOH in p53 Gene

LOH	*n*	CM1 and DO7	CM1 and p21	DO7 and p21	Ki-67 and p21
(+)	6	0.83	−0.09	0.09	0.09
(−)	21	0.70*	−0.42	−0.29	−0.72*
(+) overt AC	5	0.80	0.10	0.10	0.30
(−) overt AC	11	0.92*	−0.73***	−0.76**	−0.83**

*, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$ (Spearman's correlation coefficient).
Abbreviations: AC, adenocarcinoma.

lesions, except that combined groups of moderately and poorly differentiated adenocarcinomas tended to express this protein at lower levels than did well differentiated adenocarcinomas and AAH. Marchetti et al[22] investigated the expression of p21 in non-small cell lung carcinomas at the RNA level and by the immunohistochemistry and reported a similarly lower frequency of p21 expression in less differentiated compared with well differentiated tumors. The expression of p21 may be related to the degree of differentiation of carcinoma cells.

Importantly, in contrast to the reactive lung cells, the expression of p21 in neoplastic lung cells, including those of AAH and early adenocarcinoma, in general did not parallel p53 expression. We supposed that at least in a fraction of the neoplastic lesions and cells, p21 expression is independent of that of p53. In fact, the topographical analysis of serial sections revealed that the expression of p53 and p21 is dissociated at the cellular level in the neoplastic lesions, including AAH and early and overt adenocarcinomas. Most of overt adenocarcinomas showing high-level p53 expression expressed p21 only at a low level, again suggesting that the p53-dependent pathway was disrupted, presumably associated with loss of the normal p53 function. Lesions with high-level expression of both p53 and p21 may represent p21 expression induced or not by wild-type p53 or an accumulation of mutant p53 that retains transcriptional activity.[42]

The expression of p21 varied considerably among adenocarcinomas with LOH in the p53 gene, indicating a p53 function abnormality (Table 4). The presence of some lesions (Table 4, numbers 15 and 18) with high p21 expression and LOH in the p53 gene suggested p53-independent pathway(s) for p21 induction. Marchetti et al[22] reported that p53-independent p21 expression is a common feature of non-small cell lung carcinomas.

The mechanism underlying the p53-independent expression of p21 is unknown, but some stimuli may induce cell cycle arrest and terminal differentiation, for example growth factors.[23] Alternatively, the possibility should be borne in mind that p21 RNA may be stabilized regardless of p53 status.[21] It is unlikely that the p21 gene was mutated or amplified, since no alteration of the p21 gene has been identified in a wide variety of human neoplasms including lung cancers.[43,44]

The Ki-67 labeling index increased with the advance of lesion grade and appeared to reflect a gain of proliferative activity by the cells from premalignant to low- and high-grade malignant lung lesions. It also reflected the proliferation status of the reactive lung cells constituting interstitial pneumonia and lambertosis. However, the Ki-67 immunoreactivity in general did not correlate with p53 expression and, more importantly, with p21 expression except in overt adenocarcinomas without LOH in the p53 gene in which the expression of p21 and Ki-67 was inversely correlated. Since Ki-67 antigen is expressed in cells, except at G_0 phase, p21 may play a role in the induction of terminal differentiation in neoplastic cells with an intact p53 gene and function.

The results of this study demonstrated that in reactive lung cell proliferation the p21 expression correlated well to the p53 expression, indicating that the p53-dependent pathway was functioning normally. In contrast, in neoplastic lung cells of any grade or stage, the p21 expression was, at least in part, independent of that of p53. The expression of p21 was independent of p53 not only in overt adenocarcinomas but also in their precursors, AAH and early adenocarcinomas. These results suggest that cell cycle regulation is disrupted at a very early step in tumorigenesis of lung adenocarcinoma in which the cells seemingly harbor the wild type p53 gene. The very early disruption of cell growth control has also been demonstrated in the earliest phenotype of colonic carcinogenesis, namely, dysplastic aberrant crypt foci of colonic glands.[45] It is speculated that this dysregulation is related to a mutation of the adenomatous polyposis coli gene. The disruption of cell growth control in early pulmonary carcinogenesis demonstrated in this study may be related to an initiation event such as K-*ras* gene mutation that occurs in AAH cells at as high a frequency as that in overt adenocarcinomas.[46] Our results also suggest that the factor(s) other than p21 is important in regulating the cell cycle and maintaining terminal differentiation in normal lung cells. Additional studies are required to understand more about the mechanism of cell cycle regulation in the respiratory tract epithelium under physiologically normal and pathological conditions.

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