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Expression of cyclin D1, retinoblastoma gene protein, and p16 MTS1 protein in atypical adenomatous hyperplasia and adenocarcinoma of the lung

An immunohistochemical analysis

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Abstract To clarify the events leading to the disruption of cell growth control that occurs during the development of pulmonary adenocarcinoma (AC), we used immunohistochemistry to evaluate the expression of G1 cycle regulators, cyclin D1, Rb protein (pRb), and p16 MTS1 protein and the tumour proliferation marker, Ki 67, both in AC of the lung and in its precursor lesion, atypical adenomatous hyperplasia (AAH). The frequency of lesions with cyclin D1 overexpression was relatively high in AAH (47–89%), but was decreased in early AC (28%) and overt AC (35%). The loss of pRb expression was rare in both AAH (0–18%) and early AC (0%), and was infrequent even in overt AC (13%). The loss of p16 expression was also relatively infrequent in both the premalignant and the malignant lesions (11–25%). Our results suggest that overexpression of cyclin D1 is an early event and plays an important part in tumorigenesis in the case of lung AC. However, cyclin D1 overexpression is not required for the development and maintenance of a malignant phenotype. It is likely that some cyclin D1-independent pathways other than Rb and p16 abnormalities have an important role in the malignant transformation from AAH to early AC.

Key words Cyclin D1 · Retinoblastoma (Rb) gene protein · p16 · Atypical adenomatous hyperplasia · Adenocarcinoma of the lung

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Introduction

The arrest and progression of the cell cycle in mammalian cells is controlled through complicated pathways, and disruption of this control appears to be an important event in oncogenesis in most organs and tissues. Among numerous factors involved in cell cycle control, retinoblastoma (Rb) gene and protein (pRb) are one of the critical targets for various carcinogenic insults. Abnormalities of the Rb gene have been reported not only in hereditary and sporadic retinoblastomas but also in various types of cancers in adults including small cell lung cancer (SCLC) [6, 12], non-small-cell lung cancer (NSCLC) [33], carcinomas of the oesophagus [4], breast [43], liver [22, 50] urinary bladder [21], ovary [37], uterus [37], and prostate [10]. Abnormal Rb function is induced by either deletion or mutation of the gene in both alleles, or by binding to pRb of cellular and viral proteins. Any of these lead to the loss of normal function of pRb to bind and to inhibit the activity of transcriptional factor E2F. Cyclin D1 is a member of the cyclin family, which activates cyclin-dependent kinases (CDKs) essential for the progression of the cell cycle, mostly in the G1 phase. Cyclin D1 forms a complex with CDK4 and 6 and activates them, resulting in phosphorylation and inactivation of pRb and leading to release of E2F from it. Overexpression of the cyclin D1 protein, either with or without amplification of the gene, has been reported in various human cancers, including those of the breast [44], stomach [5] oesophagus [17], urinary bladder [32], gallbladder [42], liver [13], melanomas [41], and NSCLC [3]. p16 MTS1 plays a role in regulating the cell cycle by binding with and inhibiting the activity of CDK4 and works for pRb hypophosphorylation, so that p16 is called a CDK inhibitor (CDKI). In normal cells the level of p16 varies from extremely low or undetectable [1, 23] to high [40], depending on cell types, and maintains a complicated balance with phosphorylation of pRb or transcriptional factor levels. However, the ability of p16 to induce cell-cycle arrest is lost in cells lacking functional Rb, including primary fibroblasts from Rb–/– mouse embryos [25]. In

virally transformed cells, inactivation of pRb function activates the transcription and induces the stabilization of p16 mRNA [23, 40]. Similar results are obtained in tumour cell lines where the presence of p16 is inversely correlated with pRb [1, 23, 29, 31, 36]. The high levels of p16 in Rb-negative cells suggests that in these cells p16 competes with cyclin D1 for binding to CDK4 and CDK6 and prevents formation of active complexes, but this has not yet been proved definitively [31]. Mutations of the p16 gene have been reported in tumour cell lines and in primary tumours arising in tissues such as the pancreas [8, 24], oesophagus [28], lungs [39], and in gliomas [38].

Although it seems likely that loss of the functions of pRb and p16 and the overexpression of cyclin D1 have an important role in tumorigenesis of SCLC, NSCLC, oesophageal, breast and other cancers, little is known about their roles in the development of lung adenocarcinoma.

The prevalence of adenocarcinoma (AC) of the lung, particularly of bronchioloalveolar carcinoma, is apparently increasing in Japan, the United States, and other countries, but its pathogenesis still remains obscure. Several recent studies suggested that AC of the peripheral lung mostly develop from a precancerous lesion, atypical adenomatous hyperplasia (AAH), in a manner similar to the adenoma–carcinoma sequence in colorectal carcinogenesis [18, 19]. It has recently been reported that abnormalities of the p53–p21 pathway are implicated in lung carcinoma development [14], though their precise role still remains to be clarified.

In the present study, we used immunohistochemistry to study the expression of cyclin D1, pRb, and p16 in lung lesions at various developmental steps of peripheral lung AC to elucidate the role of their abnormalities in the tumorigenesis. We also evaluated the proliferation of premalignant and malignant cells in terms of Ki 67 expression and correlated the results with the variables mentioned above. We found that the overexpression of cyclin D1 was frequent in AAH, but was reduced during the progression from AAH to the early stage of AC (adenocarcinoma in situ), whereas the loss of pRb and p16 expression was not common in either premalignant or malignant lesions, suggesting that the loss of pRb and p16 functions does not play a significant part in the development of lung AC.

Materials and methods

Tissue samples were obtained from the surgical specimens of 34 patients who underwent lobectomy or pneumonectomy at Yokohama City University Hospital, Kanagawa Cancer Centre Hospital, Yokohama Municipal Citizen's Hospital, and Kanagawa Cardiovascular and Respiratory Centre between 1988 and 1995. Thirty-four pulmonary AC lesions were obtained from 22 patients, and 26 AAH lesions were obtained from 21 patients; 9 of these patients had concurrent AAH and AC lesions. The age of the patients (11 men, 23 women) ranged from 44 to 81 years (mean 61). The patients had received no preoperative therapy, including radiation and chemotherapy.

Tissues were fixed in 20% formalin in phosphate-buffered saline (PBS) at pH 7.4 and embedded in paraffin. Sections 4 μm thick were stained with haematoxylin and eosin for classification of lung lesions based on the grading systems of WHO and the Japan Lung Cancer Society [16, 45]. AAH were classified as low-grade (LG) AAH and high-grade (HG) AAH, according to previously described criteria [18, 19].

Sections (4 μm thick) of representative areas were used for evaluation of the expression of pRb, p16, and cyclin D1. The sections were placed on silan-coated glass slides, pretreated for antigen retrieval by microwave heating (95° C, 15 min) in citrate buffer (0.01 M, pH 6.8), washed in 0.01 M PBS, pH 7.4, and reincubated with normal goat serum. The sections were then covered with primary antibodies. The primary antibody used for detection of cyclin D1 was a polyclonal rabbit antiserum raised against C-terminal amino acids residues 285–295 of human cyclin D1 (Upstate Bio., New York, USA) diluted 1:20. The primary antibody for pRb was a mouse monoclonal antibody raised against a recombinant epitope between amino acids 612–928 of human pRb (clone 3H9, MBL, Nagoya, Japan) that detects both the phosphorylated and hypophosphorylated forms of pRb diluted 1:40. The antibody for p16 was a polyclonal rabbit antiserum (PharMingen, San Diego, USA) diluted 1:100; this antibody does not cross-react with mouse p16, but is weakly reactive with human p15 and p15.5. For detection of Ki 67 antigen, a mouse monoclonal antibody, MIB1 (Immunotech, Marseille, France), was used at 1:50 dilution. All sections were covered with primary antibodies at 4° C overnight. After washing in PBS, appropriate secondary antibodies were applied and the antigen site was detected using an avidin-biotin-peroxidase complex kit (Vector Labs, Burlingame, USA). Immunoreaction sites were visualized with 3,3´-diaminobenzidine. Sections were washed in PBS, rinsed in cold water, and lightly counterstained with haematoxylin.

Noncancerous alveolar, stromal, and inflammatory cells in the same specimens were used as positive controls for pRb, as described previously by Reissmann et al. [33]. For positive controls of cyclin D1 immunostaining, tongue and breast cancer tissues were used. Tissues of human osteosarcoma were used for positive controls of p16 immunostaining [40]. For negative controls, sections were treated in the same manner except for omission of the primary antibodies.

For each antigen, distinct nuclear staining was regarded as positive; cytoplasmic staining was disregarded. The staining results for cyclin D1 were evaluated in terms of both the intensity and frequency of positively stained cells. Tumour cell nuclei stained more intensely than those of normal epithelial cells, and the stromal cells, including inflammatory cells, were considered to show overexpression. The frequency of cells positive for cyclin D1 was determined by counting the number of cells whose nuclei were distinctly stained at any intensity among 1000 cells in randomly selected fields. Lesions with cells positive for pRb and p16 at any frequency and intensity were considered to have normal expression of pRb and p16. For pRb expression, the intensity was classified into none, weak, and intense, as compared with normal epithelial cells and the stromal cells. For Ki 67 antigen, 2000 cells were observed in the most representative areas. When the number of total cells was lower than 1000 in the entire field, all the cells were evaluated. When the internal positive controls for pRb and p16 were not clearly stained, they were excluded from the analysis.

The relationships between the staining results and various clinicopathological factors were assessed using the Chi-square test, Fisher's exact test and a two-tailed *t*-test. Differences were considered significant when the *P*-value was less than 0.05.

Results

Histopathology

LG AAH consisted of a single layer of small cuboidal cells proliferating along alveolar walls intermittently or continuously (Fig. 1a). HG AAH showed enlarged and hyperchromatic nuclei and also increased cellularity

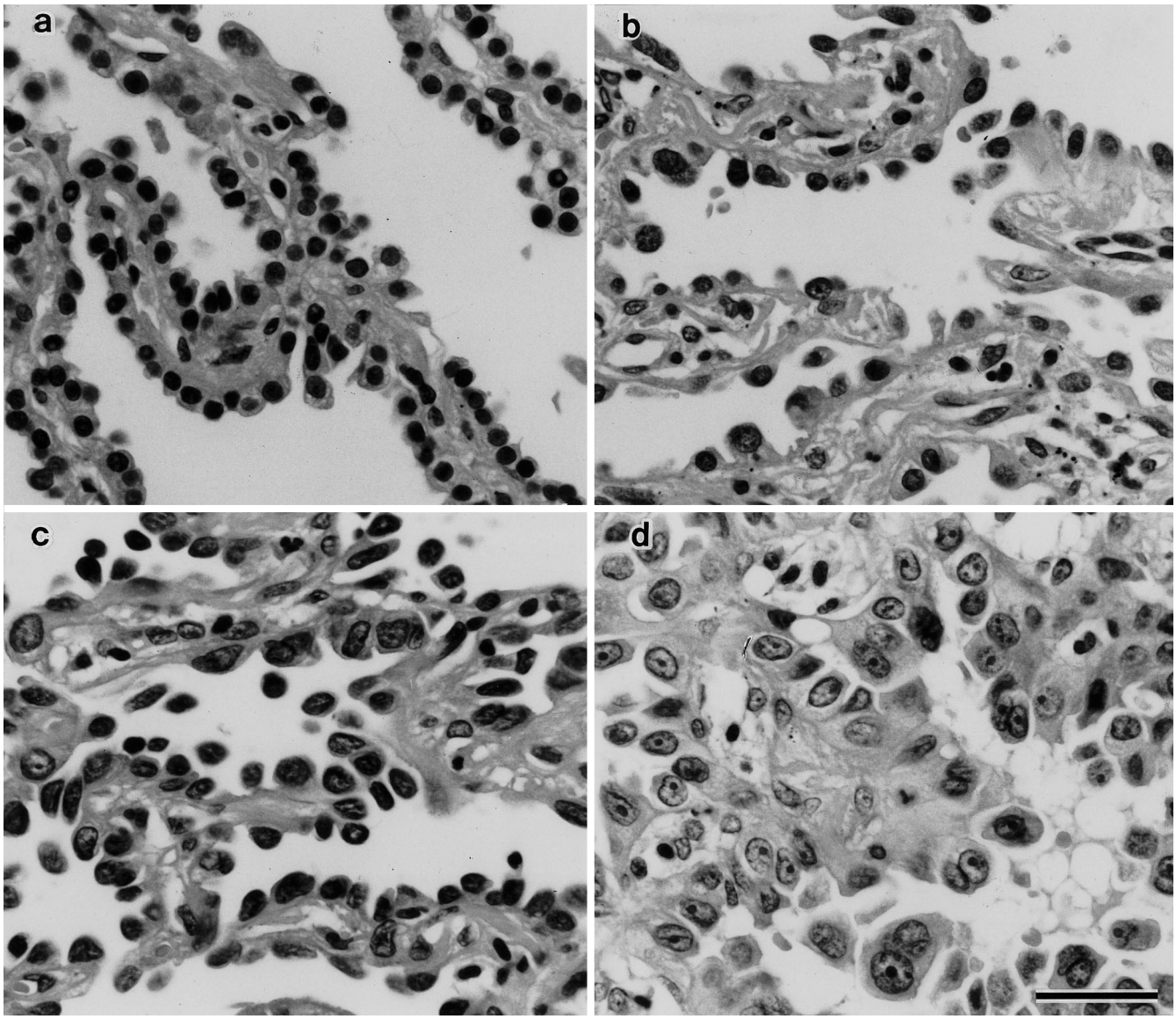


Fig. 1 Histological appearance of **a** low-grade atypical adenomatous hyperplasia (AAH), **b** high-grade AAH, **c** early adenocarcinoma (AC), and **d** overt AC. *Bar* 10 µm

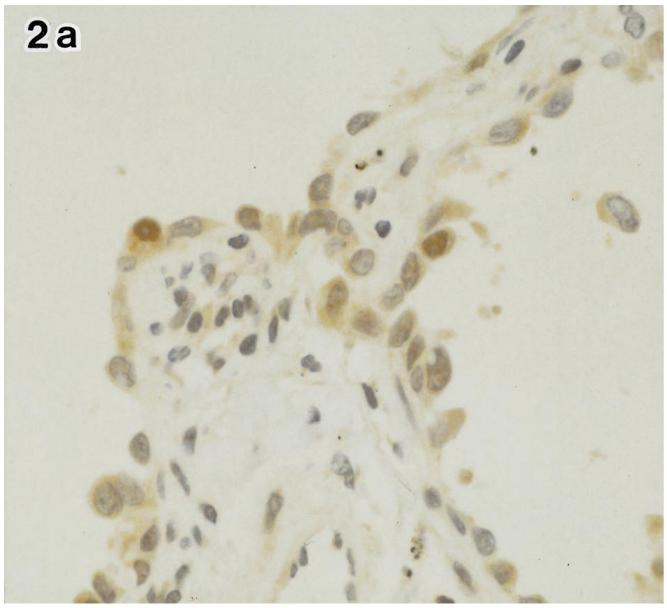
compared with LG AAH (Fig. 1b). Early AC, which is often called AC in situ in other organs, consisted of cells with nuclei that were even more hyperchromatic and pleomorphic and were sometimes stratified (Fig. 1c). Overt AC had extremely pleomorphic nuclei, and most of the cells were stratified (Fig. 1d) [18, 19]. Fifteen lesions were graded as LG AAH and 9 as HG AAH. There were 11 early AC lesions. Twenty lesions were well-differentiated and 3 were moderately-differentiated AC. All these lesions were considered on the basis of macroscopic and histological studies to have occurred in the peripheral lung. For evaluation, both well- and moderately-differentiated AC lesions were categorized as overt AC. AAH lesions ranged from 1.3 to 8.5 mm (mean 2.8 mm), early AC lesions from 6.25 to 18 mm (mean 11.0 mm),

Table 1 Incidence of lung lesions with cyclin D1 overexpression, loss of pRb, and loss of p16 (*LG AAH* low-grade atypical adenomatous hyperplasia, *HG AAH* high-grade atypical adenomatous hyperplasia, *AC* adenocarcinoma)

Category	Cyclin D1 overexpression	Loss of pRb	Loss of p16
LG AAH	7/15 (47%)[b]	3/17 (18%)	4/16 (25%)
HG AAH	8/9 (89%)[a]	0/9 (0%)	1/9 (11%)
Early AC	3/11 (28%)[b]	0/11 (0%)	2/11 (18%)
Overt AC	8/23 (35%)	3/23 (13%)	3/23 (14%)

[a] Significantly different from the corresponding values for LG AAH (P=0.004), early AC (P=0.009), and overt AC (P=0.007). P-values were calculated by Fisher's exact test

[b] Fifteen of 24 lesions (62.5%) with combined LG and HG AAH and 11 of 34 lesions (32.4%) with combined early and overt AC showed cyclin D1 overexpression, and the difference between AAH and AC was significant (P=0.017)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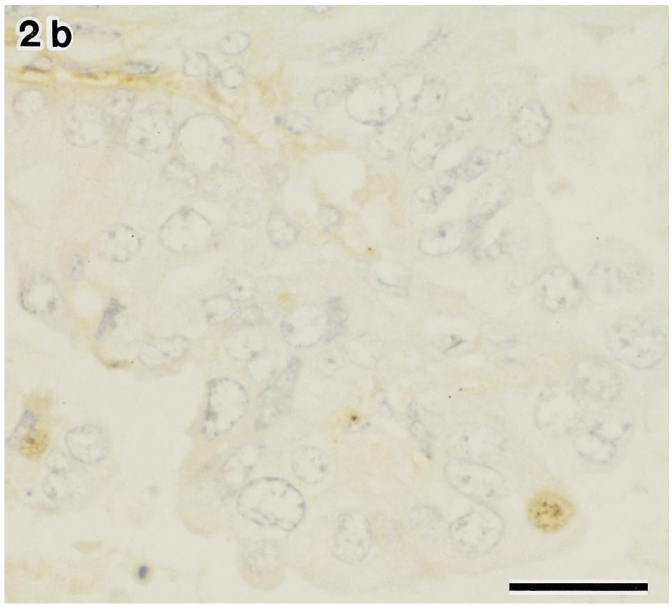
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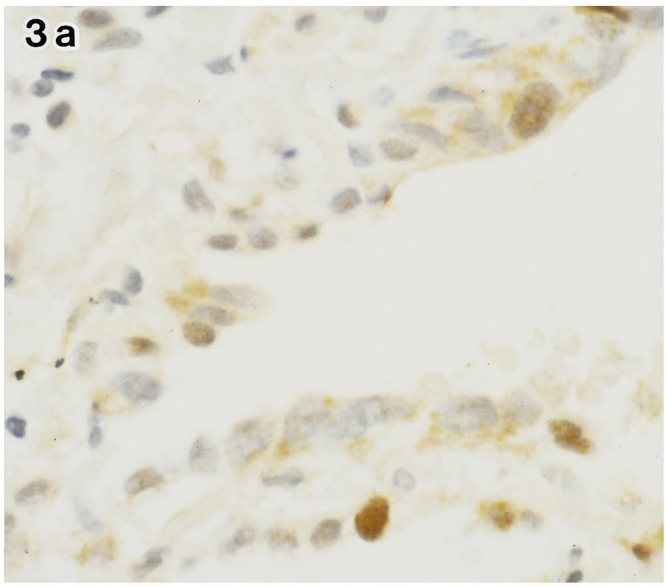
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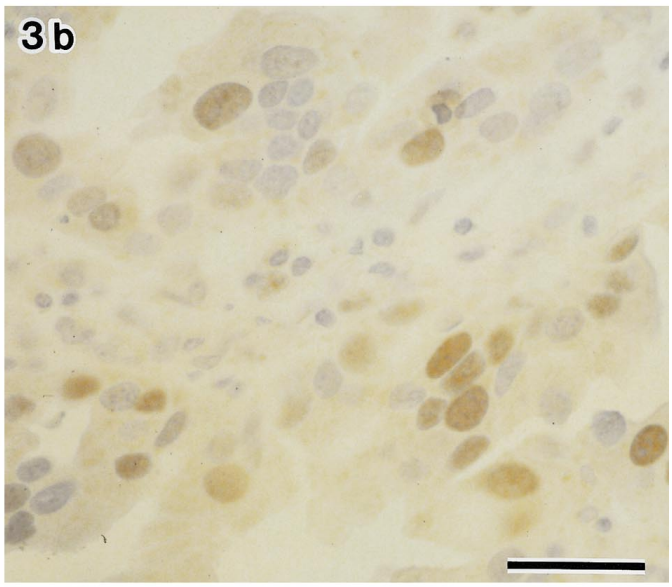
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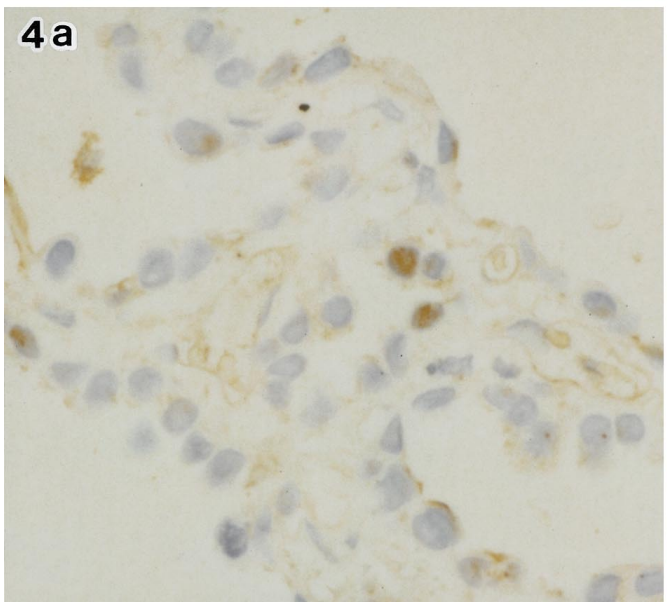
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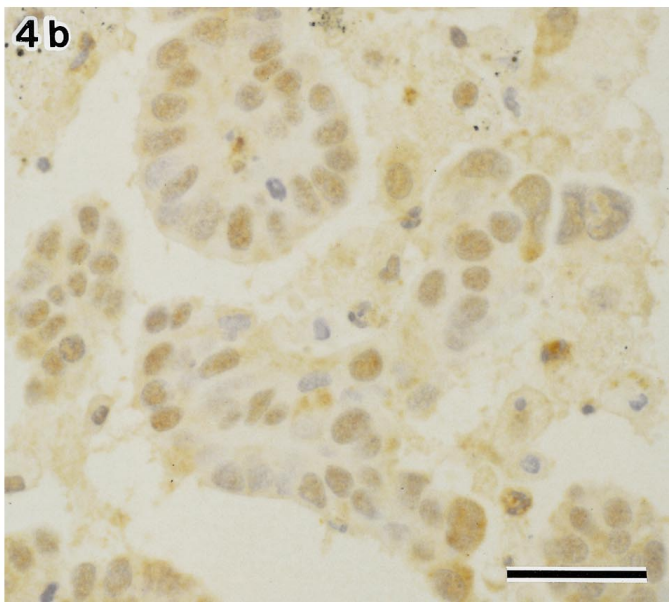
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Table 2 Frequency of occurrence of cells positive for cyclin D1 in lung lesions. *P*-values were calculated between groups indicated by Fisher's exact test

Category	Cyclin D1		
	>10% positive cells (%)	>25% positive cells (%)	>50% positive cells (%)
LG AAH (*n*=15)	12 (80)	10 (67)	6 (40)
HG AAH (*n*=9)	9 (100)	9 (100)	9 (100)
	P=0.023	*P*=0.01 *P*=0.0004	*P*=0.005
Early AC (*n*=11)	7 (64)	5 (45)	2 (18)
Overt AC (*n*=23)	14 (61)	6 (35)	4 (17)

and overt AC lesions from 10.0 to 53.0 mm (mean 24.0 mm) in size.

Immunohistochemistry

The nuclei of bronchiolar epithelial cells and type II cells in nonneoplastic lesions showed only partially and weakly positive cyclin D1 expression compared with the positive breast cancer controls. The cytoplasm of macrophages was nonspecifically positive for cyclin D1. Cyclin D1 was uniformly stained in most cyclin D1-positive AAH cells and some AC cells, but other groups of AC cells showed a granular pattern of positive reaction. The incidence of lesions with cyclin D1 overexpression is shown in Table 1. In AAH, 15/24 lesions (62.5%) showed overexpression of cyclin D1 (Fig. 2a), but only 11/34 lesions (32.4%) showed overexpression in AC (Fig. 2b); there was a significant difference between these two groups (P=0.017). Among all the categories, HG AAH showed the highest incidence of cyclin D1 overexpression (89%), which was significantly different from the corresponding values in LG AAH (P=0.004), early AC (P=0.009), and overt AC (P=0.007).

We also evaluated the cyclin D1 expression in terms of frequency of cells positive for cyclin D1 staining in each lesion and compared the results among the categories of lung lesions (Table 2). At any cutoff value used (10%, 25%, 50%), there was a clear tendency for cyclin D1 expression to be highest in HG AAH, second highest in LG AAH, and lowest in early and overt AC. Although the statistical difference between combined AAH and combined AC was most significant (P=0.0004) when the cutoff value of 25% was used, the differences was also significant at the cutoff values of 10% (P=0.023) and 50% (P=0.0005).

◀ **Fig. 2a, b** Representative immunostaining for cyclin D1. **a** In low-grade AAH cyclin D1 is more frequently and intensely expressed than in overt AC (**b**). *Bar* 10 µm

Fig. 3a, b Representative immunostaining for Rb gene protein (pRb). pRb expression is preserved in both **a** low-grade AAH and **b** overt AC. *Bar* 10 µm

Fig. 4a, b Representative immunostaining for p16. p16 expression is seen in some of the cells in both **a** low-grade AAH and **b** overt AC. *Bar* 10 µm

Immunoexpression of pRb in AAH and AC is shown in Fig. 3. pRb was expressed intensely in the nuclei of some lymphocytes and type II alveolar cells, as well as in the cytoplasm of macrophages. pRb staining was sometimes granular in AC cells. As shown in Table 1, loss of pRb expression was observed in 3/17 lesions (18%) of LG AAH, 0/9 lesions (0%) of HG AAH, 0/11 lesions (0%) of early AC, and 3/23 lesions (13%) of overt AC. Thus, 3/26 lesions (13%) of AAH lesions and 3/34 (9%) of AC lesions exhibited loss of pRb expression. There was no difference between any categories of the lung lesions.

In nonneoplastic lesions, some of the lymphocytes and type-II alveolar cells were positively stained for p16, but the staining was weaker than in positive controls. The nuclear staining of p16 in most AAH cells and some AC cells was uniform (Fig. 4a, b), but other AC cells, especially in weakly stained lesions, showed a granular pattern. The cytoplasm of the macrophages was stained nonspecifically. The cytolasm of some AC cells was also weakly stained.

There was no difference in loss of p16 expression between any category of lung lesions (Table 1). Loss of p16 expression was seen in 5/25 lesions (20%) of combined AAH and 5/34 lesions (14.7%) of combined AC.

When the staining intensity was compared between cyclin D1 and pRb, most lesions in all categories except overt AC showed intense expression of both cyclin D1 and pRb (Table 3). In overt AC, pRb expression was absent or weak in about half the lesions, and this correlated with low-level cyclin D1 expression (P=0.018).

The Ki 67 labelling index increased significantly as the malignant potential progressed (Table 4). When lesions with cyclin D1 overexpression and those with low-level expression were compared, a slight tendency was noted for the former group to have higher Ki 67 labelling index values than the latter, though the difference was not significant.

There was no correlation between cyclin D1 and p16 or between pRb and p16.

Discussion

AAH is a small lesion in the peripheral lung, exhibiting a uniform proliferation of cuboid or columnar cells along the alveolar wall. The cells of AAH show various de-

Table 3 Relationship between pRb and cyclin D1 immunostaining intensity in lung lesions

Category	pRb	n	Cyclin D1		*P*-value[a]
			none-weak	moderate-intense	
LG AAH	None–weak	5	1	4	0.495
	Moderate–intense	10	2	8	
HG AAH	None–weak	0	0	0	1.000
	Moderate–intense	9	0	9	
Early AC	None-weak	2	2	0	0.054
	Moderate–intense	9	1	8	
Overt AC	None-weak	12	7	5	0.018
	Moderate–intense	11	1	10	

[a] Calculated by Fisher's exact test

Table 4 Ki67 labelling index and levels of cyclin D1 expression

Category	*n*	Cyclin D1		
		Low-level expression	Overexpression	Total
LG AAH	15	0.72±0.80	0.74±0.98	0.73±0.86
HG AAH	9	0	1.73±1.32	1.53±1.36
Early AC	11	2.94±4.04	5.73±1.96	3.70±3.73
Overt AC	23	11.06±6.90	14.06±7.28	12.10±7.02

grees of nuclear enlargement, hyperchromatism, pleomorphism and cellularity, and these are sometimes so marked as to be hardly distinguishable from well-differentiated AC, particularly bronchioloalveolar carcinoma. Kitamura et al. have previously analysed the characteristics of AAH and distinguished it from AC by the results of morphometric and immunohistochemical evaluation of overexpression of the tumour suppressor gene p53 [19]. They showed that there was a distinct difference in p53 immunoexpression between AAH, early AC, and overt AC. More recently, Hayashi et al. reported that the regulation of the p53–p21 pathway is already disrupted in AAH [13]. From these results, it was supposed that abnormalities in G1 cycle regulation might already occur in AAH. In this study, we focused on the expression of cyclin D1 and pRb, which take positions in the lower stream of G1 cycle regulation, as well as of p16, a tumour suppressor gene product regulating the activity of pRb, since these G1 cell cycle regulators are commonly targetted in the genesis of various types of human cancer.

As AAH lesions are too small to be detected by presurgical radiological methods of diagnosis such as CT scan and are usually only found by careful pathological examination of fixed material, it is necessary to obtain as much information as possible from such small fixed samples. Furthermore, technical difficulties with tissue samples can confound blottings, because samples usually contain a mixture of contaminating inflammatory and stromal cells. For this reason, we subscribe to the previously reported view that immunohistochemical analysis of the routine samples is the most convenient method for learning the characteristics of lung lesions that we report in this study [10].

Amplification of cyclin D1 gene (PRAD1), located on 11q13, and overexpression of the protein have been reported in a subset of NSCLC including AC [3, 26]. Our results here showed the cyclin D1 was overexpressed not only in overt AC but also in early AC at frequencies comparable to those reported in previous studies. Surprisingly, cyclin D1 overexpression was much more prevalent in AAH, and particularly in HG AAH. In this study, the expression of cyclin D1 was assessed using various criteria, as in studies reported hitherto many different criteria have been employed [2, 3, 26, 44]. All the results were, as a rule, consistent, in that cyclin D1 overexpression was more frequent in AAH than in AC, most frequent in HG AAH, and equal in early AC and overt AC. Overexpression of cyclin D1 in premalignant lesions has been reported in murine [49] and human [2, 49] colonic carcinogenesis [2, 49] and in murine skin carcinogenesis [34]. In addition, Koshikawa et al. reported results similar to those shown here in their study of AAH and AC [20]. It thus appears that overexpression of cyclin D1 is a common and presumably important event that occurs in the earlier stages of carcinogenesis in many tissues, including the peripheral lung. However, it is likely that cyclin D1 overexpression is no longer required for maintenance of malignant phenotype once the lesions have become malignant, at least in lung carcinogenesis. A similar reduction of cyclin D1 expression has been reported in a study on human breast cancers, where a negative correlation of cyclin D1 expression with proliferative activity and differentiation markers was demonstrated [44]. A cyclin D1-independent proliferation pathway, for example one related to a defect in the Rb gene, is considered a plausible explanation for this observation [44]. Alternatively, increased

levels of cyclin D1 can be found not only in growth-promoting but also in growth-arresting conditions [9]. Thus it can be speculated that overexpression of cyclin D1 in AAH cells may reflect a growth-inhibitory state against growth-promoting effects exerted by some unknown mechanism.

Abnormality of the Rb gene is commonly seen in wide spectrum of human tumours. While SCLC shows a high frequency of loss of pRb expression [6] and LOH of the gene [12], NSCLC preserves the Rb gene and function relatively well [33]. In particular, AC shows a lower frequency of loss of pRb expression (10–32%) [33, 47] than other types of NSCLC. The low incidence of loss of pRb expression in overt AC shown here was comparable to that in previous reports. No HG AAH or early AC showed loss of pRb expression, suggesting that loss of Rb occurs at the step of progression from early AC to overt AC in a subset of this type of tumour. A small fraction of LG AAH lesions showed no pRb expression. Although precise molecular and biochemical analysis is required, it is plausible that almost all the cells in these LG AAH lesions were in the G0 phase, during which pRb is not expressed [46].

The abnormality of p16 at DNA level is mostly a homozygous deletion. The abnormality of MTS1 gene and loss of p16 protein are seen in specific tumours including lung cancers, where p16 abnormalities are significantly different between SCLC and NSCLC. In SCLC, p16 abnormality is rarely found, presumably because of the high frequency of Rb gene abnormality in this type of tumour. In contrast, p16 is undetectable in a substantial fraction (70%) of NSCLC cell lines, but the frequency of p16 gene mutations detected by DNA sequencing in primary NSCLC is lower and ranges from 0% to 30% [7, 15, 30]. This discrepancy may result from in vitro culture conditions, significant contamination of non-cancerous cells in tumour samples, abolition of p16 gene expression by mutations in p16 non-coding regions, methylation of the CpG island surrounding the first exon of p16 [27, 39], or decreased stability of p16 protein [35]. In fact, methylation of p16 has been reported in 26% of AC lesions, but SCLC has rarely shown evidence for methylation [27, 39]. These reports suggest that the immunohistochemical approach is suitable for investigating p16 dysfunction at the protein level, as emphasized by Geradts et al. [11]. Our results showed that loss of p16 expression was relatively infrequent not only in AAH but also in AC and that the frequency of loss in the two types was nearly equal. Sakaguchi et al. have reported that the frequency of loss of p16 was 37% in AC [36], a figure comparable to that of our present study. Our observations that there was no significant difference between AAH and AC are consistent with the assumption that mutation of the p16 gene is a late event in NSCLC carcinogenesis because a high rate of mutation was detected in metastatic NSCLC [30].

The Ki 67 labelling index was significantly increased with the advance of lesion grade. However, there was only a slight positive correlation with cyclin D1 immunoexpression. These results suggest that tumour cell proliferation and cyclin D1 overexpression do not necessarily parallel each other.

In conclusion, since the overexpression of cyclin D1 was relatively high in AAH, but was decreased during progression from AAH to early stage of AC, cyclin D1 overexpression appears to be important in the early stage of lung AC oncogenesis. pRb and p16 were well preserved in both the premalignant and the malignant lesions, and thus apparently do not have an important role in the development of lung AC.

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References

1. Aagaard L, Lukas J, Bartkova J, Kjerulff A-A, Strauss M, Bartek J (1995) Aberrations of $p16^{INK4}$ and retinoblastoma tumour-suppressor genes occur in distinct sub-sets of human cancer cell lines. Int J Cancer 61:115–120
2. Arber N, Hibshoosh H, Moss SF, Sutter T, Zhang Y, Begg M, Wang S, Weinstein IB, Holt PR (1996) Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis. Gastroenterology 110:669–674
3. Betticher DC, Heighway J, Hasleton PS, Altermatt HJ, Ryder WDJ, Cerny T, Thatcher N (1996) Prognostic significance of CCND1 (cyclin D1) overexpression in primary resected non-small-cell lung cancer. Br J Cancer 73:294–300
4. Boynton RF, Huang Y, Blount PL, Reid BJ, Rashkind WH, Haggitt RC, Newkirk C, Resau JH, Yin J, McDaniel T, Meltzer SJ (1991) Frequent loss of heterozygosity at the retinoblastoma locus in human esophageal cancers. Cancer Res 51: 5766–5769
5. Buckley MF, Sweeney KJE, Hamilton JA, Sini RL, Manning DL, Nicholson RI, deFazio A, Watts CKW, Musgrove EA, Sutherland RL (1993) Expression and amplification of cyclin genes in human breast cancer. Oncogene 8:2127–2133
6. Cagle PT, El-Naggar AK, Xu H-J, Hu S-X, Benedict WF (1997) Differential retinoblastoma protein expression in neuroendocrine tumors of the lung. Potential diagnostic implications. Am J Pathol 150:393–400
7. Cairns P, Shaw ME, Knowles MA (1993) Initiation of bladder cancer may involve deletion of a tumour-suppressor gene on chromosome 9. Oncogene 8:1083–1085
8. Caldas C, Hahn SA, da Costa LT, Redston MS, Schutte M, Seymour AB, Weinstein CL, Hruban RH, Yeo CJ, Kern SE (1994) Frequent somatic mutations and homozygous deletions of the p16 (*MTS1*) gene in pancreatic adenocarcinoma. Nat Genet 8:27–32
9. Chen X, Bargonetti J, Prives C (1995) p53, through p21 (WAF1 /CIP1), induces cyclin D1 synthesis. Cancer Res 55: 4257–4263
10. Geradts J, Hu S-X, Lincoln CE, Benedict WF, Xu H-J (1994) Aberrant *RB* gene expression in routinely processed, archival tumor tissues determined by three different anti-RB antibodies. Int J Cancer 58:161–167
11. Geradts J, Kratzke RA, Niehans GA, Lincoln CE (1995) Immunohistochemical detection of the cyclin-dependent kinase inhibitor 2/multiple tumor suppressor gene 1 (*CDKN2 / MTS1*) product $p16^{INK4A}$ in archival human solid tumors: correlation with retinoblastoma protein expression. Cancer Res 55: 6006–6011
12. Harbour JW, Lai S-L, Whang-Peng J, Gazdar AF, Minna JD, Kaye FJ (1988) Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. Science 241: 353–357

13. Hatada I, Tokino T, Ochiya T, Matsubara K (1988) Co-amplification of integrated hepatitis B virus DNA and transforming gene *hst-1* in a hepatocellular carcinoma. Oncogene 3:537–540
14. Hayashi H, Miyamoto H, Ito T, Kameda Y, Nakamura N, Kubota Y, Kitamura H (1997) Analysis of $p21^{Waf1/Cip1}$ expression in normal, premalignant, and malignant cells during the development of human lung adenocarcinoma. Am J Pathol 151:461–470
15. Hayashi N, Sugimoto Y, Tsuchiya E, Ogawa M, Nakamura Y (1994) Somatic mutations of the MTS (multiple tumor suppressor) 1/CDK4I (cyclin-dependent kinase-4 inhibitor) gene in human primary non-small cell lung carcinomas. Biochem Biophys Res Commun 202:1426–1430
16. Japan Lung Cancer Society (1995) General rule for clinical and pathological record of lung cancer, 4th edn. The Society, Tokyo
17. Jiang W, Kahn SM, Tomita N, Zhang Y-J, Lu S-H, Weinstein B (1992) Amplification and expression of the human cyclin D gene in esophageal cancer. Cancer Res 52:2980–2983
18. Kitamura H, Kameda Y, Nakamura N, Nakatani Y, Inayama Y, Iida M, Noda K, Ogawa N, Sibagaki T, Kanisawa M (1995) Proliferative potential and p53 overexpression in precursor and early stage lesions of bronchioloalveolar lung carcinoma. Am J Pathol 146:876–887
19. Kitamura H, Kameda Y, Nakamura N, Inayama Y, Nakatani Y, Sibagaki T, Ito T, Hayashi H, Kimura H, Kanisawa M (1996) Atypical adenomatous hyperplasia and bronchoalveolar lung carcinoma. Analysis by morphometry and the expressions of p53 and carcinoembryonic antigen. Am J Surg Pathol 20: 553–562
20. Koshikawa T, Yatabe Y, Nakamura S, Nishio M, Toyama G, Yokoi T, Kato K, Sato T (1996) Immunohistochemical observation of cyclin D1, p53, Rb, SPA, S100a and S100b in precursor lesions of bronchioloalveolar carcinoma of the lung. Jpn J Chest Dis 55:799–805
21. Kubota Y, Miyamoto H, Noguchi S, Shuin T, Kitamura H, Xu H-J, Hu S-X, Benedict W (1995) The loss of retinoblastoma gene in association with c-myc and transforming growth factor-β1 gene expression in human gladder cancer. J Urol 154: 371–374
22. Kuroki T, Fujiwara Y, Nakamori S, Imaoka S, Kanematsu T, Nakamura Y (1995) Evidence for the presence of two tumour-suppressor genes for hepatocellular carcinoma on chromosome 13q. Br J Cancer 72:383–385
23. Li Y, Nichols MA, Shay JW, Xiong Y (1994) Transcriptional repression of the D-type cyclin-dependent kinase inhibitor p16 by the retinoblastoma susceptibility gene product pRb. Cancer Res 54:6078–6082
24. Liu Q, Yan Y-X, McClure M, Nakagawa H, Fujimura F, Rustgi AK (1995) MTS-1 (CDKN2) tumor suppressor gene deletions are a frequent event in esophagus squamous cancer and pancreatic adenocarcinoma cell lines. Oncogene 10:619–622
25. Lukas J, Parry D, Aagaard L, Mann DJ, Bartkova J, Strauss M, Peters G, Bartek J (1995) Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature 375:503–510
26. Mate JL, Ariza A, Aracil C, López D, Isamat M, Pérez-Piteira J, Navas-Palacios JJ (1996) Cyclin D1 overexpression in non-small cell lung carcinoma: correlation with Ki 67 labelling index and poor cytoplasmic differentiation. J Pathol (Lond) 180: 395–399
27. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, Sidransky D (1995) 5´ CpG island methylation is associated with transcriptional silencing of the tumour suppressor *p16/CDKN2/MTS1* in human cancers. Nat Med 1: 686–692
28. Mori T, Miura K, Aoki T, Nishihira T, Mori S, Nakamura Y (1994) Frequent somatic mutation of the *MTS1/CDK4*I (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma. Cancer Res 54: 3396–3397
29. Okamoto A, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP, Forrester K, Gerwin B, Serrano M, Beach DH, Harris CC (1994) Mutations and altered expression of $p16^{INK4}$ in human cancer. Proc Natl Acad Sci USA 91: 11045–11049
30. Okamoto A, Hussain SP, Hagiwara K, Spillare EA, Rusin MR, Demetrick DJ, Serrano M, Hannon GJ, Shiseki M, Zariwala M, Xiong Y, Beach DH, Yokota J, Harris CC (1995) Mutations in the *p16 INK4/MTS1/CDKN2*, *p15 INK4B/MTS2*, and *p18* genes in primary and metastatic lung cancer. Cancer Res 55: 1448–1451
31. Parry D, Bates S, Mann DJ, Peters G (1995) Lack of cyclin D–Cdk complexes in Rb-negative cells correlates with high levels of $p16^{INK4/MTS1}$ tumour suppressor gene product. EMBO J 14:503–511
32. Proctor AJ, Coombs LM, Cairns JP, Knowles MA (1991) Amplification at chromosome 11q13 in transitional cell tumours of the bladder. Oncogene 6:789–795
33. Reissmann PT, Koga H, Takahashi R, Figlin RA, Holmes EC, Piantadosi S, Cordon-Cardo C, Slamon DJ, the lung cancer study group (1993) Inactivation of the retinoblastoma susceptibility gene in non-small-cell lung cancer. Oncogene 8:1913–1919
34. Robles AI, Conti CJ (1995) Early overexpression of cyclin D1 protein in mouse skin carcinogenesis. Carcinogenesis 16: 781–786
35. Rusin MR, Okamoto A, Chorazy M, Czyzewski K, Harasim J, Spillare EA, Hagiwara K, Hussain SP, Xiong Y, Demtrick KJ, Harris CC (1996) Intragenic mutations of the *p16* INK4, *p15* INK4B and *p18* genes in primary non-small-cell lung cancers. Int J Cancer 65:734–739
36. Sakaguchi M, Fujii Y, Hirabayashi H, Yoon H-E, Komoto Y, Oue T, Kusafuka T, Okada A, Matsuda H (1996) Inversely correlated expression of p16 and Rb protein in non-small cell lung cancers: an immunohistochemical study. Int J Cancer 65: 442–445
37. Sasano H, Comerford J, Silverberg SG, Garrett CT (1990) An analysis of abnormalities of the retinoblastoma gene in human ovarian and endometrial carcinoma. Cancer 66:2150–2154
38. Schmidt EE, Ichimura K, Reifenberger G, Collins VP (1994) *CDKN2* (*p16/MTS1*) gene deletion or *CDK4* amplification occurs in the majority of glioblastoma. Cancer Res 54: 6321–6324
39. Shapiro GI, Park JE, Edwards CD, Mao L, Merlo A, Sidransky D, Ewen ME, Rollins BJ (1995) Multiple mechanisms of $p16^{INK4A}$ inactivation in non-small cell lung cancer cell lines. Cancer Res 55:6200–6209
40. Tam SW, Shay JW, Pagano M (1994) Differential expression and cell cycle regulation of the cyclin-dependent kinase 4 inhibitor $p16^{INK4}$. Cancer Res 54:5816–5820
41. Theillet C, Le Roy X, De Lapeyrière O, Grosgeorges J, Adnane J, Raynaud SD, Simony-Lafontaine J, Goldfarb M, Escot C, Birnbaum D, Gaudray P (1989) Amplification of *FGF*-related genes in human tumors: possible involvement of *HST* in breast carcinomas. Oncogene 4:915–922
42. Tsuda T, Nakatani H, Tahara E, Sakamoto H, Terada M, Sugimura T (1989) *HST1* and *INT2* gene coamplification in a squamous cell carcinoma of the gallbladder. Jpn J Clin Oncol 19: 26–29
43. Tsukamoto K, Ito N, Yoshimoto M, Iwase T, Tada T, Kasumi F, Nakamura Y, Emi M (1996) Two distinct commonly deleted regions on chromosome 13q suggest involvement of BRCA2 and retinoblastoma genes in sporadic breast carcinomas. Cancer 78:1929–1934
44. Van Diest PJ, Michalides RJAM, Jannink I, van der Valk P, Peterse HL, de Jong JS, Meijer CJLM, Baak JPA (1997) Cyclin D1 expression in invasive breast cancer. Correlations and prognostic value. Am J Pathol 150:705–711
45. World Health Organization (1981) International histological classifications of tumours. 1. Histological typing of lung tumours, 2nd edn. WHO, Geneva
46. Xu H-J, Hu S-X, Benedict WF (1991) Lack of nuclear RB protein staining in G0/middle G1 cells: correlation to changes in total RB protein level. Oncogene 6:1139–1146

47. Xu H-J, Quinlan DC, Davidson AG, Hu S-X, Summers CL, Li J, Benedict WF (1994) Altered retinoblastoma protein expression and prognosis in early-stage non-small-cell lung carcinoma. J Natl Cancer Inst 86:695–699
48. Yeager T, Stadler W, Belair C, Puthenveettil J, Olopade O, Reznikoff C (1995) Increased p16 levels correlate with pRb alterations in human urothelial cells. Cancer Res 55:493–497
49. Zhang T, Nanney LB, Luongo C, Lamps L, Heppner KJ, DuBois RN, Beauchamp RD (1997) Concurrent overexpression of cyclin D1 and cyclin-dependent kinase 5 (Cdk4) in intestinal adenomas from multiple intestinal neoplasia (Min) mice and human familial adenomatous polyposis patients. Cancer Res 57:169–175
50. Zhang X, Xu H-J, Murakami Y, Sachse R, Yashima K, Hirohashi S, Hu S-X, Benedict WF, Sekiya T (1994) Deletions of chromosome 13q, mutations in *Retinoblastoma 1*, and retinoblastoma protein state in human hepatocellular carcinoma. Cancer Res 54:4177–4182