Virchows Arch (1996) 428:159–163

ORIGINAL ARTICLE

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Immunohistochemistry of cyclin D3 in pulmonary carcinomas

Received: 2 October 1995 / Accepted: 12 February 1996

Abstract Cyclin D3, a cell cycle regulator, is encoded in the 6q21 chromosome region. Abnormalities of this gene and its protein product have not been found in normal tissues or in malignancies from human subjects. The expression of cyclin D3 was studied immunohistochemically in archival formalin-fixed, paraffin-embedded specimens from normal organs obtained from three autopsy cases and 237 human primary pulmonary carcinomas. In normal organs, nuclear positivity for cyclin D3 was observed in reactive type-2 pneumocytes, islets of Langerhans, lymphocytes from lymph nodes, superficial cells of transitional epithelium, epithelium of oesophagus, stomach, small intestine and gallbladder, endothelium, smooth muscles, and brain. Proliferating cells such as lymphocytes in the germinal centres and non-proliferating cells such as neurons both demonstrated cyclin D3 immunoreactivity. Cyclin D3 showed obvious nuclear immunoreactivity in 168 pulmonary carcinomas (71%). The proportion of tumour cells that were cyclin D3-positive ranged from 1% to 73% (median, 16%). There was no relationship between cyclin D3 immunoreactivity and histological typing, tumour differentiation, or pathological TNM staging. In pulmonary carcinomas, distinct expression of the cyclin D3 protein is unlikely to be implicated in tumorigenesis, because of its expression in only a small fraction of cancer cells. It may relate to cancer progression. The distribution of cyclin D3 reactivity in the normal tissues suggests that cyclin D3 affects other processes than cell cycle regulation in a lineage-specific manner.

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Key words Cyclin D3 · Immunohistochemistry · Pulmonary carcinoma

Introduction

Cyclins are proteins that control cell cycle progression and cell growth by regulating cdc2 kinase and other related kinases [16]. Five classes of mammalian cyclins (A, B, C, D, and E) have been identified. Type-D cyclins are synthesized early in the G1 phase, and they regulate G1/S transition. There are three types of D cyclin (D1, D2, and D3) with sequence homology expressed, at least partially depending on cell lineages [5, 17, 24, 25, 27, 28, 32, 37]. Several studies have suggested that individual D-type cyclins have distinct functions in different cell types, and that their biological roles may well not overlap [2, 11, 20, 32].

Cyclin D1, D2, and D3 genes were mapped to chromosome regions 11q13, 12p13 and 6p21, respectively [17, 37]. It has been proposed that cyclin D1 and D2 are proto-oncogenes, but the cyclin D3 gene has not yet been identified as a proto-oncogene. Rearrangement of the cyclin D1 gene has been reported in parathyroid adenomas [26] and in B cell lymphomas [30, 35], while gene amplification occurs in a subset of other malignancies, including carcinomas of the breast, oesophagus, head and neck, liver and lung [1, 7–9, 12, 13, 18, 19, 22, 29, 31, 33, 34, 38, 39]. The cyclin D2 gene is also amplified in some colorectal carcinomas [23]. These genetic alterations are associated with increased expression of cyclins, and several immunohistochemical studies of cyclin D1 have been performed on archival paraffin-embedded cancer tissues [3, 4, 6, 7, 13, 38]. Only tumour cells have been demonstrated to exhibit cyclin D1 immunoreactivity. To our knowledge, no data from immunohistochemical studies of cyclin D3 in human cancer tissues have been published in the literature. In this study, we examined the expression of cyclin D3 in 237 archival formalin-fixed, paraffin-embedded specimens of human primary lung cancer by immunohistochemistry.

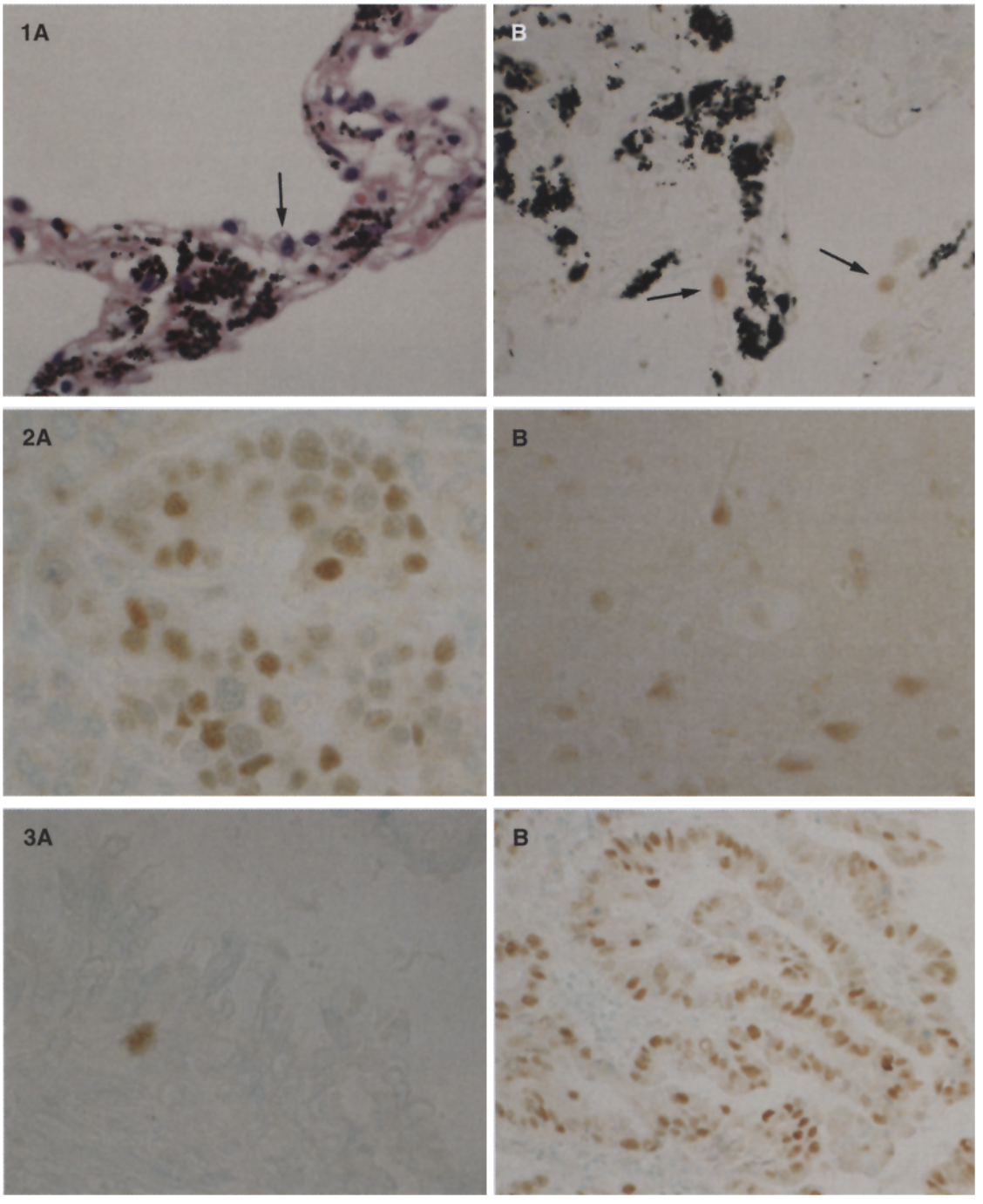

Fig. 1A, B Nuclear positivity for cyclin D3 in reactive type II pneumocytes (*arrows*). **A** ×200, **B** ×200

Fig. 2 Cyclin D3 immunoreactivity **A** in the Langerhans island of pancreas (×200) and **B** in the neural cells of cerebral cortex (×200)

Fig. 3A, B Immunohistochemical staining of cyclin D3 in primary pulmonary carcinomas, adenocarcinomas (**A** ×200, **B** ×200). Cyclin D3 immunoreactivity is localized to the nuclei of cancer cells. The proportion of cyclin D3 positive cells in carcinomas is 1% and 62% in **A** and **B**, respectively

Materials and methods

The materials in this study consisted of archival 10% formalin-fixed and paraffin-embedded blocks from normal organs removed from three patients at autopsy and from 237 lungs that were surgically resected as treatment for primary carcinoma between November 1984 and December 1994.

The normal organs examined were as follows: 1 brain, 1 hypophysis, 3 salivary glands, 2 thyroid glands, 2 tonsils, 3 lymph nodes, 2 hearts, 2 lungs, 2 oesophagi, 3 stomachs, 3 small and large intestines, 2 livers, 1 gallbladder, 2 pancreata, 2 kidneys, 2 adrenal glands, 3 urinary bladders, 1 testis, 1 prostate gland, 2 uteri, and 2 ovaries.

The patients with pulmonary carcinoma were male in 186 and female in 51. Median patient age was 66 years, with a range from

20 to 82 years. The resected lungs were immediately fixed by the bronchial perfusion method with 10% formalin. The blocks, from the Second Department of Pathology of Niigata University School of Medicine, were prepared from 119 adenocarcinomas, 94 squamous cell carcinomas, 1 adenosquamous cell carcinoma, 8 large cell carcinomas, 10 small cell carcinomas, 3 carcinoid, 1 adenoid cystic carcinoma, and 1 mucoepidermoid carcinoma. In each case, the sample included the tumour and the surrounding non-tumour-bearing tissue. From each sample, 3-μm-thick paraffin sections were prepared and stained with haematoxylin-eosin. The pathological features of the specimens were classified and staged according to World Health Organization criteria [36] and the TNM staging system [14].

A mouse monoclonal antibody raised against human cyclin D3 (DCS-22; Novocastra Laboratories, Newcastle, UK) was used for immunostaining. Sections of 3 μm thick were used. After deparaffinization and blocking of endogenous peroxidase activity in the sections, the microwave oven heating technique [10] was applied. In brief, the sections were placed in plastic jars filled with citrate buffer (2.1 g citric acid monohydrate/l destilled water, pH was adjusted to 6.0 with NaOH) and incubated 7 times for 3 min at 500 W in a household microwave oven (model KRF-1004; Tiger, Osaka, Japan). After heating, the sections were allowed to cool to room temperature, which took approximately 20 min, and rinsed in 0.05 mol/l phosphate-buffered saline (PBS). A modified immunoglobulin–enzyme bridge technique (avidin–biotin–peroxidase complex method) was used for immunostaining. The sections were incubated with 10% normal rabbit serum for 15 min to block non-specific conjugation in the tissue. Subsequently, they were incubated with the primary anti-cyclin D3 monoclonal antibody at a dilution of 1:40 for 12 h at 4°C. After washing with PBS, the sections were incubated with biotinated rabbit anti-mouse immunoglobulin [Histofine SAB-PO (M) Kit, Nichirei Corporation, Tokyo, Japan] for 30 min at room temperature, followed by washing with PBS and incubation with peroxidase-conjugated streptavidin [Histofine SAB-PO (M) Kit] for a further 30 min. Immune conjugates were visualized with 0.05 mol/l TRIS-hydrochloric acid (pH 7.6) containing both 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride and 0.03% (v/v) hydrogen peroxide. Negative control sections from each staining run were made using normal mouse IgG instead of the anti-cyclin D3 antibody.

Distinct nuclear cyclin D3 immunoreactivity was judged a positive result. In each specimen of lung cancer, more than 1000 tumour cells were counted from random fields, using a ×40 objective magnification, to estimate the percentages of positive cells. Statistical analysis was done by non-parametric methods. Differences between the two groups were determined by the Mann-Whitney U-test. The significance level was set at $P<0.05$, and all tests were two-sided.

Results

Reactive type 2 pneumocytes showed nuclear staining for cyclin D3 (Fig. 1A, B). Cyclin D3 reactivity was found in the normal organs (Table 1). Nuclear positivity was observed in islets of Langerhans (Fig. 2A), lymphocytes of lymph nodes, and superficial cells of transitional epithelium, although staining intensity varied. In the lymph nodes, positive lymphocytes were present mainly in the germinal centres. Several epithelial cells showed

Table 1 Distribution of cyclin D3 immunoreactivity on various human organs. + Positive immunoreactivity (nuclear), – negative

Oesophagus		Gallbladder	+[a]	Fibroblast	–
Squamous mucosa	+[a]	Pancreas		Cartilage	–
Gastric mucosa		Duct	–	Endothelium	+[a]
Foveola	+[a]	Acinar cell	–	Fatty tissue	–
Fundic gland	–	Langerhans island	+	Tonsil	–
Pyloric gland	+[a]	Kidney		Lymph node	+[e]
Intestinal mucosa		Gomerulus	–	Uterus	
Goblet cell	+[a]	Tubule	–	Cervical epithelium	–
Paneth cell	–	Adrenal gland		Endometrial gland	–
Colonic mucosa	–	Cortex	–	Stroma	–
Lung		Medulla	–	Ovary	–
Bronchial mucosa	–	Transitional epithelium	+[c]	Salivary gland	–
Bronchial gland	–	Prostate	–	Thyroid gland	–
Pneumocyte	+[b]	Seminiferous tubule	–	Brain	
Liver		Heart	–	Neural cell	+[a]
Liver cell	–	Skeletal muscle	–	Glial cell	+[a]
Bile duct	–	Smooth muscle	+[d]		

[a] Several cells
[b] Only reactive type II pneumocytes
[c] Mainly in the superficial cells
[d] Nuclear and cytoplasmic reactivity
[e] Mainly in the germinal centers

Table 2 Immunoreactivity of cyclin D3 in pulmonary carcinomas

Histological typing (no. of cases)	No. of positive cases (%)	Mean relative number of cyclin D3 immunoreactivity in positive cases (range)
Adenocarcinoma (119)	99 (83%)	19% (1–73)
Well differentiated (58)	50 (86%)	20% (1–73)
Moderately differentiated (22)	21 (95%)	21% (1–63)
Poorly differentiated (39)	28 (72%)	17% (1–66)
Squamous cell carcinoma (95)	56 (59%)	13% (1–72)
Well differentiated (11)	8 (73%)	15% (1–42)
Moderately differentiated (61)	36 (59%)	13% (1–72)
Poorly differentiated (23)	12 (53%)	12% (1–45)
Large cell carcinoma (8)	7 (88%)	12% (1–60)
Small cell carcinoma (10)	5 (50%)	2% (1–5)
Carcinoid (3)	1 (33%)	8%
Adenoid cystic carcinoma (1)	0 (0%)	0%
Mucoepidermoid carcinoma (1)	0 (0%)	0%

Table 3 Relationship between cyclin D3 immunoreactivity and pathological TNM staging

Pathological TNM (no. of cases)	No. of positive cases (%)	Mean relative number of cyclin D3 immunoreactivity in positive cases (range)
T1 (77)	58 (75%)	15% (1–67)
T2 (107)	79 (74%)	15% (1–72)
T3 (28)	17 (61%)	21% (1–66)
T4 (25)	14 (56%)	23% (1–73)
N0 (132)	95 (72%)	12% (1–72)
N1 (43)	31 (72%)	18% (1–73)
N2 (55)	37 (67%)	22% (1–63)
N3 (7)	5 (71%)	37% (1–62)
M0 (216)	154 (71%)	16% (1–73)
M1 (21)	14 (67%)	19% (1–47)

cyclin D3 reactivity in the oesophagus, stomach, small intestine, and gallbladder. Positive nuclear staining for cyclin D3 was also found in several endothelial cells of various organs and neural and glial cells of brain (Fig. 2B). Smooth muscle demonstrated nuclear and cytoplasmic reactivity for cyclin D3.

Cyclin D3-positive tumour cells were observed in 168 of the 237 primary lung cancer samples (71%). In the majority of cancers examined, staining was localized to the nucleus and the intensity of the reaction varied from cell to cell (Fig. 3A, B). The proportion of cyclin D3-positive cells ranged from 1% to 73%, with a median of 16%. The frequency of mitosis in the cancerous tissue varied regardless of the degree of cyclin D3 immunoreactivity. In some cases, cancer cells exhibited cytoplasmic staining.

There was no relationship between cyclin D3 immunoreactivity and histological typing, tumour differentiation, and pathological TNM staging (Tables 2, 3).

Discussion

Immunohistochemical staining of cyclin D1 has been used in the analysis of many types of carcinoma [3, 4, 6, 7, 13, 19, 38, 39], and it has usually shown a good correlation with gene amplification [13, 19, 39]. In breast cancer, distinct nuclear cyclin D1 immunoreactivity was identified in cases with and without gene amplification [13, 38]. Overexpression of cyclin D1 occurs not only by way of gene amplification, but also by way of chromosomal rearrangement [15, 30, 35]. DNA was not available from the archival formalin-fixed, paraffin-embedded specimens in this study; however, abnormalities of cyclin D3, including amplification and rearrangement, have not been reported in human carcinomas.

In this study, we analysed the expression of cyclin D3 in lung carcinomas, and for comparison normal tissues were also tested. Using a monoclonal antibody, we found nuclear staining in neoplastic and non-neoplastic tissue. In normal organs, cyclin D3 was positive immunohistochemically not only in proliferating cells, such as germinal centres [10], but also in non-proliferating cells such as neurons. Cyclin D3 may have other functions than cell cycle regulation that it discharges in a lineage-specific manner. Cyclin D3-positive nuclei were found in 71% of the cases of lung carcinoma, but the number of positive cancer cells was small in many tumours. Aberrant nuclear overexpression or accumulation of the cyclin D3 protein is thus unlikely to be implicated in the genesis of pulmonary carcinomas.

Although the mechanisms by which cyclins act are controversial, in vitro cyclins stimulate cdk4 (cyclin-dependent kinase)-mediated phosphorylation of the retinoblastoma protein (Rb), which inactivates the growth-suppressive properties of Rb [11, 15, 21]. Rb can interact more efficiently with cyclin D2 and D3 than with D1 in vitro [11]. Unregulated phosphorylation of Rb in response to overexpressed cyclin D3 might therefore lead to a loss of growth control. Abnormally elevated expression of cyclin D3 may contribute to inappropriate cell division and tumour growth. However, the mitotic index was not always proportional to the rates of cyclin D3 expression in tumour cells. Furthermore, expression of cyclin D3 was not related to histological type, tumour differentiation, or pathological TNM staging. These results suggest that cyclin D3 expression may not reflect the biological malignancy of lung tumours directly. Although it is possible that cyclin D3 reactivity in the cancers examined was the result of abnormality in the gene or expression control system of cyclin D3, it seems to be a late event in cancer evolution.

In conclusion, pulmonary carcinomas showed distinct nuclear expression of cyclin D3. However, there was no obvious relationship between cyclin D3 expression and the histopathological features of lung cancer. Further studies on the relationships between cyclin D3 expression, patient survival rates, and alterations of the cyclin D3 gene may provide useful information on the significance of cyclin D3 in lung cancer.

Acknowledgement We wish to thank Mr. K. Ohyachi for technical assistance.

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