Prognostic significance of *CCND1* (cyclin D1) overexpression in primary resected non-small-cell lung cancer

DC Betticher[1,4], J Heighway[1], PS Hasleton[3], HJ Altermatt[4], WDJ Ryder[5], T Cerny[4] and N Thatcher[2]

CRC Departments of [1]Cancer Genetics and [2]Medical Oncology, Paterson Institute for Cancer Research, Christie Hospital (NHS) Trust, Wilmslow Road, Manchester M20 9BX, UK; [3]Pathology Department, Wynthenshawe Hospital, Manchester; [5]Medical Statistics, Christie Hospital, Manchester; [4]University Hospital of Berne, Switzerland.

Summary Amplification of the *CCDN1* gene encoding cyclin D1 was examined by Southern blotting and multiplex polymerase chain reaction (PCR) and occurred in 8 of 53 patients (15%) with primary resected non-small-cell lung cancer (NSCLC). These tumours and 17 additional tumours with a normal gene copy number showed overexpression of cyclin D1 (25/53, 47%), as assessed by immunostaining using a monoclonal antibody. In 22/25 cases, cyclin D1 was localised in the cytoplasm, but some (7/25) had simultaneous nuclear staining. This result is in marked contrast to that reported in breast, hepatocellular and colorectal carcinoma studies where immunostaining was invariably nuclear. Examination of a restriction fragment length polymorphic (RFLP) site within the 3'untranslated region of the cDNA following reverse transcriptase (RT)-PCR (29/53 informative cases) showed a strong association between cytoplasmic staining and imbalance in allele-specific message levels. Cyclin D1 overexpression was associated with a poorly differentiated histology ($P = 0.04$), less lymphocytic infiltration of the tumour ($P = 0.02$) and a reduction in local relapse rate ($P = 0.01$). The relative risk of local relapse was 9.1 in tumours without cyclin D1 overexpression ($P = 0.01$, Cox regression analysis). We conclude that genetic alteration of cyclin D1 is a key abnormality in lung carcinogenesis and may have diagnostic and prognostic importance in the treatment of resectable NSCLC.

Keywords: non-small-cell lung carcinoma; *CCND1*; cyclin D1; prognostic factor; local relapse; cell cycle regulation

Advances in molecular biology paved the way for the identification of proteins responsible for the regulation of cell proliferation. One of these molecules, cyclin D1, is expressed during the early phase of the cell cycle, before the restriction point (START), beyond which cells are committed to enter S phase. This cyclin may act as an initiator of the cell cycle, and perhaps as a growth factor sensor (Xiong *et al.*, 1991; Motokura and Arnold, 1993*a,b*; Sherr, 1993; Hunter and Pires, 1994; Marx, 1994). The ability of cyclin D1 to immortalise cells *in vitro* established its oncogenic activity (Hinds *et al.*, 1994; Lovec *et al.*, 1994). The cyclin D1 gene (*CCND1*) is one of the most frequently amplified chromosomal regions (11q13) in human carcinomas (Motokura and Arnold, 1993*b*). In laryngeal (Jares *et al.*, 1994) and head and neck carcinomas (Müller *et al.*, 1994; Michalides *et al.*, 1995), its overexpression has been shown to be associated with advanced local invasion and presence of lymph node metastases. Cyclin D1 may therefore play a key role in cell growth regulation and tumorigenesis.

Lung cancer is a worldwide problem and in many countries it is the most lethal malignancy. As relapse is frequent after resection of early stage non-small-cell lung cancer (NSCLC) (Ginsberg *et al.*, 1993), there is an urgent need to define prognostic factors. These would help in the choice of the best therapeutic procedure (wide surgical resection, (neo-)adjuvant chemo- and/or radiotherapy). In this study we will provide evidence that, surprisingly, cyclin D1 overexpression is associated with lower local relapse risk in resectable NSCLC, despite also being associated with poor tumour differentiation, known to be a negative indicator.

Patients and methods

Patients' characteristics and tumour specimens

Tumour samples were obtained from 53 consecutive patients [49 men, four women, median age 64 years (45-79)] who

underwent resection of staged resectable NSCLC at the University Hospital of Berne, Switzerland. They had received no therapy before surgery [pneumonectomy ($n = 15$) or lobectomy ($n = 38$)]. Twenty-nine patients were in stage 1, five in stage 2 and 19 in stage 3 (UICC, 1987). All tumours were classified according to the standard criteria of the WHO (1981), by two pathologists, independently (HJA, PSH): there were 35 squamous carcinomas, 11 adenocarcinomas, four undifferentiated NSCLC, two large-cell carcinomas and one carcinoid. Tumour size was measured by the pathologists on the fresh specimen. The lymphocytic infiltration of the tumour, the amount of necrosis and vascular infiltration were determined histologically. Thirty-three of 53 patients are alive with a median follow-up of 16 months (range 2-42). In order to examine the possibility whether the immunostaining result was due to fixation artifacts we analysed also 33 NSCLC tumour sections prepared in the Wythenshawe Hospital for cyclin D1 overexpression.

DNA and RNA extraction

DNA and RNA were isolated from frozen tissue samples. High molecular weight DNA was prepared according to standard protocols (Blin *et al.*, 1976) and RNA was extracted using Dynabeads (Dynal, Oslo, Norway) (Jakobsen *et al.*, 1990) before being reverse transcribed to cDNA (Superscript Preamplification System, Life Technologies, Paisley, UK).

Southern blot analysis

*Taq*I digests of 5 μg of genomic DNA were blotted onto Hybond-N filter (Amersham, Buckinghamshire, England). Purified DNA probes (*CCND1*, nt: 1100-1888 and adenosine deaminase, ADA, assigned to chromosome 20q12-13, nt: 34275-34785) were labelled with α-^{32}P-dCTP (Amersham) using a random-primed DNA labelling kit (Boehringer, Mannheim, Germany) and hybridised overnight to the immobilised DNA. Filters were subsequently washed for 30 min, once with 2 × SSC and once with 0.5 × SSC at 65°C and exposed to Kodak X-OMAT film at −70°C for several days. The *CCND1* gene was considered amplified when the

intensity of the ratio *CCND1*/ADA signal (assessed by Phosphor Imager analysis, 425S, Molecular Dynamics) was increased at least 3-fold relative to the ADA gene signal, which served as an internal control for DNA loading and which was assumed not to be amplified in lung tumours. Digested DNA isolated from normal lung tissue from each patient was used as a control.

PCR

The PCR multiplex was performed as described (Edwards and Gibbs, 1994). The *CCND1* gene (chromosome 11q13) was compared with the adenosine deaminase (ADA) gene (chromosome 20q12–13) and the progesterone receptor (PR) gene (chromosome 11q23). The following primers were used: *CCND1*: 5'ATATTCCGTAGGTAGATGTGTAAC 3', 5'TGTCACTATTTCGTCTTCTC 3'; ADA: 5'GCGGGTG-AACGTCAATGTGTTC 3', 5'CAACCTGAAGAGAGT-GTGCAAG 3'; PR; 5'GGTTTGTTTCTCACTCATATAGC 3', 5'GTAGGACCTCAAGGTGTAGC 3'. Genomic DNA (2 μg) was added to 98 μl of a PCR mixture containing dNTP at 250 μM, 0.5 μg of each *CCND1* and 0.05 μg ADA primers or 0.17 μg PR primers (the ADA and PR primer concentrations were reduced in order to obtain an equal amplification compared with *CCND1* in normal lung tissue), 1 × PCR buffer (Boehringer) and 2 U *Taq* polymerase (Boehringer). PCR cycles (30) were run in an automated thermocycler: denaturation 94°C, 1 min, annealing 57°C, 1 min, and elongation temperature 74°C, 1 min. PCR products were visualised on 2% agarose gels. For the RFLP analysis of the 3'UTR of the cyclin D1 genomic and cDNA the following primers were used: primary PCR (30 cycles), only for cDNA: 5'ACAACTTCCTGTCCTACTACCG3', 5'ATAGTAGCG-TATCGTAGGAGTG 3' and secondary PCR (genomic primary, 30 cycles) cDNA analyses (1 μl of a 1:100 dilution of primary PCR amplification, 28 cycles) 5'-CTCTTGGTTA-CAGTAGCGTAGC3', 5'-ATCGTAGG-AGTGGGACAG-GT-3'. The PCR products were digested with the restriction enzyme *Hae*III and visualised on 2% gels. Allelic imbalance in DNA/cDNA derived products was determined visually with reference to normal control DNA samples.

Immunohistochemistry

The immunohistochemistry was performed as described previously (Gillett et al., 1994). Briefly, formalin-fixed paraffin sections (4 μM) from the 53 primary lung tumours prepared in Berne, 33 in Manchester and 14 breast cancers were air dried on 2% 3-aminopropyltriethoxysilane (APTS) (Sigma, Poole, UK)-coated slides. After dewaxing in xylene, the sections were treated for 15 min with 300 ml methanol and 10 ml hydrogen peroxide in order to block endogenous peroxidase and rinsed thoroughly in water. They were then placed in citrate buffer, boiled twice in a microwave, washed with water and placed in TRIS buffer (pH 7.6). After incubation in 1:100 goat serum for 20 min at room temperature, they were placed in 1% bovine serum albumin (BSA) and 1:100 monoclonal cyclin D1 antibody (Novocastra, Newcastle, England) overnight at room temperature. After two washes with TRIS buffer they were incubated in 1:100 biotinylated goat anti-mouse/rabbit IG(Dako, Glostrop, Denmark) for 30 min, washed subsequently twice with TRIS, incubated in 1:100 solution of streptavidin–biotin complex for 30 min at room temperature, rewashed in TRIS buffer and placed in diaminobenzene (10 mg 10 ml⁻¹) for 10 min, counterstained with haematoxylin, washed in water, cleared, dehydrated and mounted. Cyclin D1 staining was examined according to the intensity of the majority of cells. The slides were assessed blind, and the pathologists did not know the results of the molecular analyses. Positive and negative controls were performed for each tumour series. Two categories of staining were used: nil–weak, and moderate–strong. A tumour was considered positive in the case of a moderate–strong staining. In some cases of negative cyclin D1 tumour staining we repeated the analyses in several regions of the tumour.

Statistical analyses

Patients were placed into two groups according to their cyclin D1 expression. Associations of group membership with other patient and tumour characteristics were made with chi-square tests for categorical features and Mann–Whitney *U*-tests for continuous ones. Disease end points analysed were overall survival and event-free survival (EFS) (defined as the time to the first of: (i) local relapse with or without metastases; (ii) metastases alone; (iii) death from an unrelated cause). Kaplan–Meier survivor function estimates were used and simple comparisons between the two groups were made with the log-rank test. EFS is a mixing of three types of event (Kay et al., 1983) and the type-specific cumulative event rates were estimated with the Nelson estimator (Nelson, 1969). Cox regression (Cox, 1972) was used to investigate if the group difference with respect to the local relapse rate remained after adjustment for age, T-stage, N-stage and tumour differentiation.

Results

Amplification of the CCND1 gene in NSCLC

Fifty-three patients with operable NSCLC were investigated for *CCND1* gene amplification by Southern blotting and multiplex PCR analyses. In 8/53 (15%) of the NSCLC a 3- to 20-fold amplification of the *CCND1* gene was identified (5/35 squamous cell, 2/11 adeno- and 1/2 large cell carcinoma, Figure 1). This result was confirmed by a PCR multiplex comparing *CCND1* amplification with the amplification of the adenosine deaminase gene (chromosome 20q12–13), and numerical differences in chromosome 11 copy number were excluded by a PCR multiplex, comparing *CCND1* with the progesterone receptor gene located on the same arm of chromosome 11 (Figure 1). Five patients with amplification were informative for the cyclin D1 gene polymorphism (Heighway, 1991); allelic imbalance was observed in each case supporting the amplification data obtained by Southern blotting and PCR multiplex.

Cyclin D1 expression analysis by immunostaining

Tumour sections from the 53 patients were stained using a cyclin D1 monoclonal antibody. Following the protocol kindly provided by C Gillett and D Barnes (Gillett et al., 1994) we found cyclin D1 overexpression in 6 out of 14
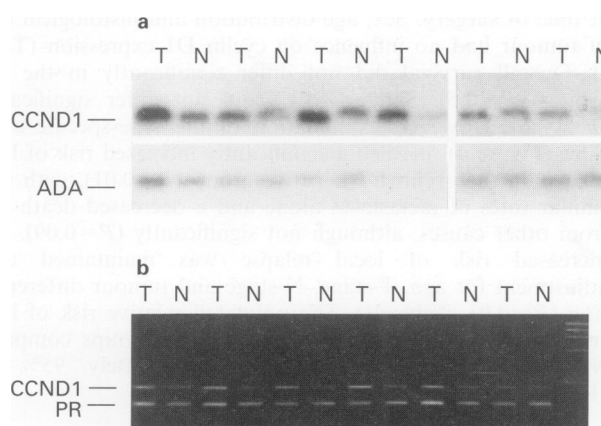
Figure 1 (a) Southern blot: Tumour (T) and adjacent normal lung tissue DNA blot hybridised with the *CCND1* and adenosine deaminase (control) probes. The lanes were loaded with a total of 5 μg of *Taq*1-digested DNA. All tumours demonstrate a 3-to 20-fold increase in *CCND1* copy number compared with adjacent non-tumour tissue. (b) PCR multiplex analyses: PCR amplification of *CCND1* and progesterone receptor gene (chromosome 11q23) showed increase of *CCND1* copy number compared with control genes, confirming the Southern analysis and excluding an aberrant copy number of chromosome 11.

breast tumours, which was mainly confined to the nucleus. In our 53 lung tumours moderate–strong staining occurred at a similar overall frequency (25/53, 47%). All tumours with amplification of the CCND1 gene demonstrated overexpression of cyclin D1. Additionally, 17 tumours (32%) with an apparently normal gene copy number also showed increased cyclin D1 protein levels. However, in contrast to breast tumours, the cyclin D1 intracellular distribution varied. Simultaneous nuclear and cytoplasmic staining was observed in 7/53 (13%) cases, and specific cytoplasmic or nuclear staining was seen in 15/53 (28%) and 3/53 (6%) cases respectively (Figure 2). Tumours with CCND1 amplification showed nuclear ($n = 3$), cytoplasmic ($n = 3$) or both, nuclear and cytoplasmic staining ($n = 2$). In order to exclude a fixation artifact we assessed the cyclin D1 overexpression in 33 NSCLC tumours fixed in the Wythenshawe Hospital. A very similar result (nuclear staining 2/33, cytoplasmic 6/33 and nuclear and cytoplasmic 3/33) was obtained in those patients, rendering this explanation rather unlikely. The cause and significance of this altered subcellular localisation, however, is not clear and cyclin D1 expression was further investigated using RT–PCR.

Allele-specific cyclin D1 expression

A polymorphism of cyclin D1 located in the 3' untranslated region allows a PCR-based determination of allele-specific expression within RNA samples by RFLP analysis (Heighway, 1991). Twenty-nine out of the 53 tumour samples were informative for this polymorphism. All informative patients with cyclin D1 overexpression ($n = 14$) had an imbalance in allele-specific expression levels suggesting up-regulation of one parental gene, consistent with the immunostaining results (Figure 3). However, in the case of the three tumours with both CCND1 amplification and cytoplasmic cyclin D1 staining, the allele imbalance was present but paradoxically, the amplified allele appeared to show reduced expression relative to the unamplified allele. As it is reasonable to assume that the amplified allele is in fact overexpressed, these results suggest that the majority of CCND1 mRNA encoded from this allele may be spliced in a way precluding PCR amplification of the cDNA with the primers necessary to visualise the RFLP.

Association of histopathological findings and clinical outcome

Tumours with cyclin D1 overexpression were less differentiated ($P = 0.04$) and showed less lymphocytic infiltration ($P = 0.02$). There was no difference in stage and tumour size at time of surgery. Sex, age distribution and histological type of tumour had no influence on cyclin D1 expression (Table I). Overall survival did not differ significantly in the two groups ($P = 0.5$). Similarly EFS did not differ significantly ($P = 0.26$). However, examination of the type-specific event rates (Figure 4) revealed a significantly increased risk of local relapse in the cyclin D1-negative group ($P = 0.01$), with very similar rates of metastases alone and a decreased death rate from other causes, although not significantly ($P = 0.09$). The increased risk of local relapse was maintained after adjustment for age, T-stage, N-stage and tumour differentiation ($P = 0.01$, Table II). The estimated relative risk of local relapse was 9.1 in the cyclin D1-negative groups compared with the positive group with approximately 95% CI (1.5,55.4).
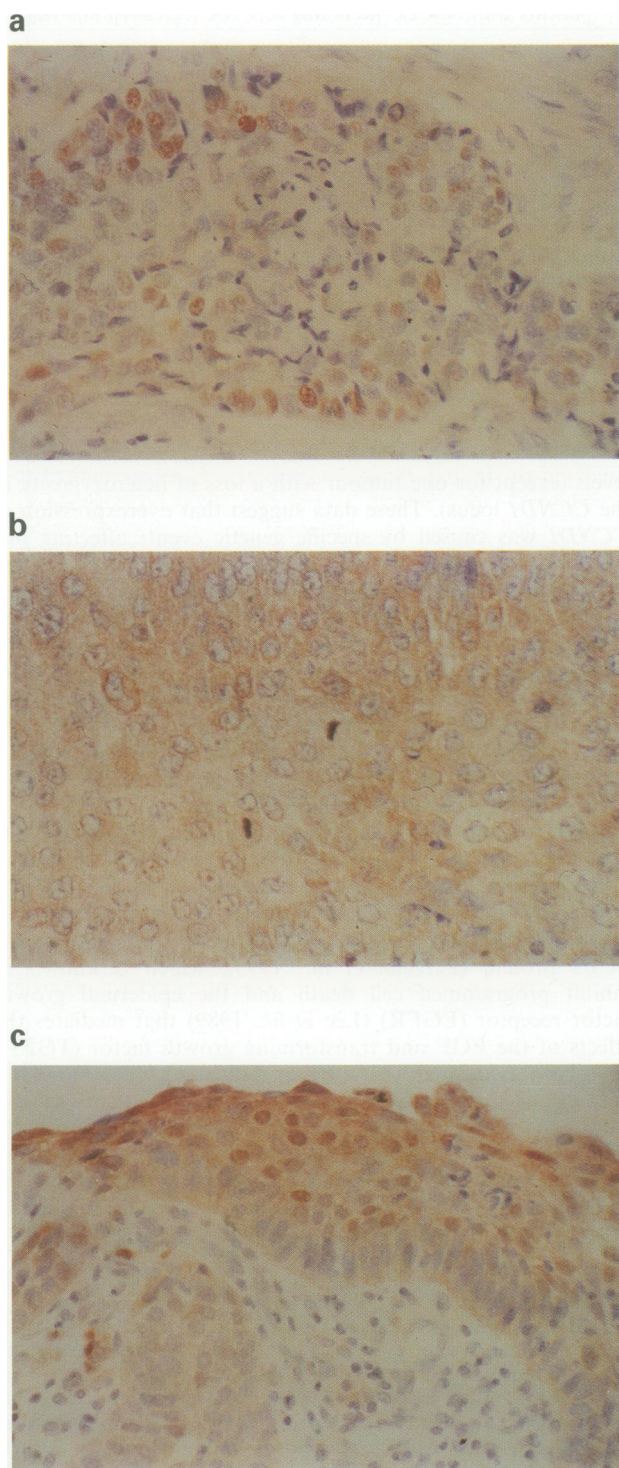
Discussion

G_1 cyclins such as cyclin D1 and their cyclin-dependent kinases (CDKs) are proving to be integrators of growth factor-mediated signals driving cells through the restriction point (START), early in the G_1 phase of the cell cycle (Pines, 1993; Sherr, 1993, 1994). Growth factors act by binding to specific cell-surface receptors, which in turn trigger signalling cascades that ultimately govern the transcription of genes

important in cell growth. The cyclin D1 gene, CCND1, was originally identified as the proto-oncogene (PRAD1), clonally rearranged with the parathyroid hormone gene, in parathyroid adenomas (Motokura et al., 1991). Its ability to rescue G_1 cyclin-defective yeast (Lew et al., 1991; Xiong et al., 1991) suggested that this protein was a cell-cycle regulator. In addition, the murine homologue of cyclin D1, CYL1, isolated from mouse macrophages was induced by colony-stimulating factor 1 (CSF-1) (Matsushime et al., 1991). In human cell lines, cyclin D1 is stimulated by mitogens during G_1, and, upon persistent growth factor stimulation, it is continuously synthesised throughout the cell cycle (Sewing et al., 1993). However, it executes its critical function during mid-to-late G_1 phase, as cells cross the first cell cycle restrction point (for review see Motokura and Arnold, 1993b; Hunter and Pines, 1994; Sherr, 1995).

Several lines of evidence suggest that cyclin D1 might play a crucial role in carcinogenesis. Firstly, in addition to its involvement in parathyroid adenomas, in centrocytic B cell lymphomas (mantle cell lymphomas) CCND1 expression is up-regulated under the influence of the immunoglobulin heavy-chain gene enhancer, as a result of a reciprocal chromosomal translocation at the BCL1 breakpoint, t(11;14)(q13;q32) (Withers et al., 1991; Seto et al., 1992; de Boer et al., 1993). Overexpression of cyclin D1 has been demonstrated in almost all lymphoproliferative disorders carrying this translocation (Bosch et al., 1994; Lukas et al., 1994; de Boer et al., 1995; Delmer et al., 1995). Secondly, the CCND1 gene has been shown to be frequently amplified and overexpressed in many tumours, such as breast (Lammie et al., 1991; Schuuring et al., 1992; Gillett et al., 1994), head and neck (Williams et al., 1993; Callender et al., 1994; Müller et al., 1994), laryngeal (Jares et al., 1994), oesophageal (Jiang et al., 1992; Jiang et al., 1993; Kanda et al., 1994; Adélaide et al., 1995), hepatocellular (Zhang et al., 1993; Nishida et al., 1994) and lung (Shapiro et al., 1995) carcinomas.

The role of cyclin D1 in carcinogensis has been strengthened by in vitro studies demonstrating its oncogenic potential. Constitutive overexpression in rodent cells can shorten G_1 phase (Quelle et al., 1993). Similarly, in breast cancer cells arrested in G_1, cyclin D1 induction is sufficient to complete a cell cycle and shortens G_1/S phase (Musgrove et al., 1994). Conversely, microinjection of cyclin D1 antibodies or antisense plasmid into dividing cells blocks them in the G_1 phase (Baldin et al., 1993; Quelle et al., 1993). Transfection of CCND1 expression constructs into normal fibroblasts stimulates proliferation by reduction of the G_1/S (Quelle et al., 1993). Consistent with this, by complementing a defective adenovirus E1A oncogene, cyclin D1 can immortalise cells (Hinds et al., 1994), and finally when transfected into normal fibroblasts with activated HRAS, it promotes the formation of fibrosarcomas in nude mice (Lovec et al., 1994). In conclusion, there is no doubt that cyclin D1 may act as an oncogene.
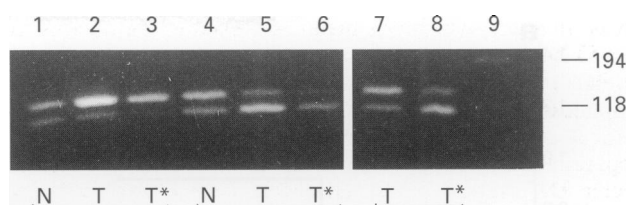
The mechanism of action of cyclin D1 is not fully established and is still a field of intensive research. Nevertheless, one clue to its function comes from the fact that the retinoblastoma protein (pRb) is down-regulated in an undulating fashion in late G_1 (Wiman, 1993), and that the pRb inactivation (permitting cell proliferation) occurs predominantly by phosphorylation of its threonine and serine residues. Interactions between cyclin D1 and other cell-cycle regulators, such as cyclin-dependent kinases (CDK4, CDK6) and the interaction of cyclin D1/CDK4 complexes with p16 (CDK4) have recently been suggested to play an important role in lung tumorigenesis (Shapiro et al., 1995). There are now accumulating data, showing MTS1 (the gene encoding p16) missense mutations and deletions with probable consequent loss of p16 function in some tumour types. In oesophageal carcinomas the mutation rate was 21–50% (Mori et al., 1994; Zhou et al., 1994), in head and neck carcinomas 10% (Zhang et al., 1994) and in NSCLC 10–30% (Hayashi et al., 1994). In several lung cancer cell lines and tumour specimens, the presence of p16 protein is inversely correlated with detectable pRb and cyclin D1 proteins (Serrano et al., 1993; Shapiro et al., 1995). In

Figure 3 RFLP analysis of *CCND1* PCR products from three patients. Normal lung DNA from patient (N) (lanes 1 and 4), NSCLC DNA (T) (lanes 2, 5, 7) and cDNA (T*) (lanes 3, 6, 8): Lanes 1–3, 4–6, the amplified allele is overexpressed (both tumours showed nuclear cyclin D1 staining), lanes 7–8, tumour DNA shows no allelic imbalance but transcript levels are clearly imbalanced (cytoplasmic cyclin D1 overexpression); lane 9, phiX174 *Hae*III marker (Promega).

Table I Association of cyclin D1 overexpression and patient characteristics

	Cyclin D1 not overexpressed	Cyclin D1 overexpressed	P-value
Number of patients	28	25	
Sex			P=0.15
Male	24	25	
Female	4	0	
Age (years)			P=0.94
Range	48–78	45–79	
Median	63.5	65	
Mean ± SD	64.1 ± 7.1	63.3 ± 9.0	
Histology			P=0.99
Squamous carcinoma	19	16	(squamous vs
Adenocarcinoma	4	7	others)
Large cell	0	2	
Carcinoid	1	0	
NSCLC	4	0	
Stage			P=0.33
1	18	11	
2	2	3	
3	8	11	
Surgical intervention			P=0.73
Lobectomy	19	19	
Pneumonectomy	9	6	
Tumour size (cm)			P=0.68
Range	1–9.3	2–8.6	
Median	4.0	4.0	
Mean ± SD	4.3 ± 2.1	4.4 ± 1.7	
missing values	3	1	
Tumour differentiation			P=0.04
Good	10	2	
Intermediate–poor	18	23	
Lymphocytic infiltration of the tumour			P=0.02
Poor	10	18	
Moderate to important	18	7	

Figure 2 Cyclin D1 immunostaining of NSCLC with monoclonal antibody (Novocastra, Newcastle, UK). (a) Nuclear staining. (b) Cytoplasmic staining. (c) Simultaneous nuclear and cytoplasmic staining.

particular, cells lacking functional pRb have no cyclin D1/CDK4 complexes (Bates et al., 1994), and in NSCLC cell lines, p16 was absent when pRb function was normal and present when pRb was mutated (Otterson et al., 1994). These results highlight the importance of the cyclin D1–CDK4–p16 pathway in tumorigenesis.

A monoclonal cyclin D1 antibody allows the detection of cyclin D1 by immunohistochemistry, enabling a clear distinction to be made between different levels of expression. Gillet et al. (1994) have found an excellent correlation between *CCND1* gene amplification and cyclin D1 overexpression with this antibody. Immunostaining of sections from the eight lung tumours with *CCND1* amplification
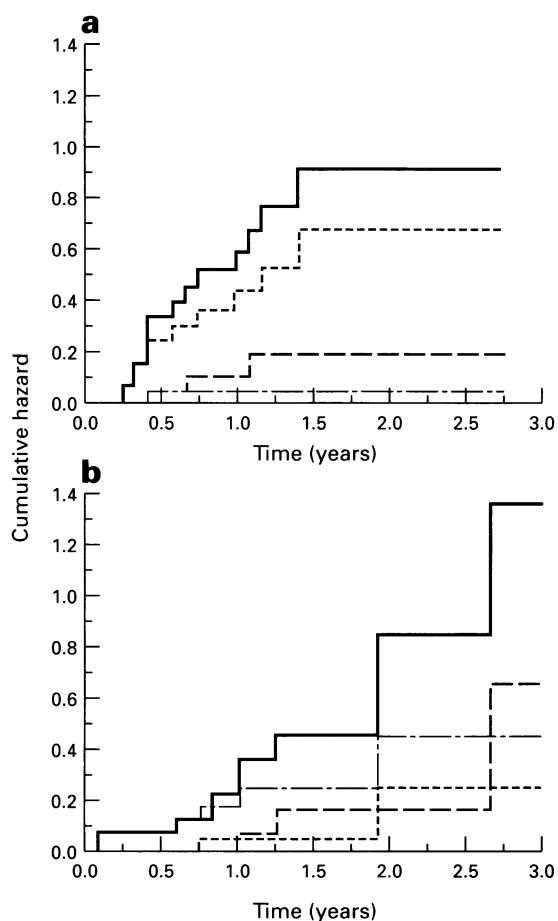
showed that all overexpressed the protein. High-level expression was also seen in a further 17 cases in which amplification of the gene could not be demonstrated. In contrast to previous studies on other malignancies (Jiang et al., 1993; Zhang et al., 1993; Bartkova et al., 1994, 1995; Gillett et al., 1994)) in which cyclin D1 was confined to the nucleus, in our study, cyclin D1 protein was frequently observed at high levels, and sometimes exclusively, within the cytoplasm of the tumour cells. This finding is in agreement with the very recently published report (Lukas et al., 1995) on cyclin D1 and D2 in in vitro growing U-2-OS cells showing subcellular cytoplasmic localisation, during the G_1/S phase while nuclear was observed during the G_1 phase. The authors suggest a possible change of solubility of the protein dependent on the cell-cycle phase. Others have also reported cytoplasmic or cytoplasmic+nuclear cyclin D1 staining in mantle cell lymphoma (Banno et al., 1994; Nakamura et al., 1994). Different contrasting functions (positive regulators of growth in combination with CDK4 and CDK6, but an

Factor	Variable	Estimate	s.e.	Relative risk	P-value
Cyclin D1	0 Positive				
	1 Negative	2.212	0.901	9.1	0.01
Age (years)	0– <65				
	1– ≥65	–0.489	0.742	0.6	0.50
Stage	0 1–2				
	1 3	1.234	1.536	3.4	0.41
Lymph node	0 No				
metastases	1 Yes	–0.047	1.550	1.0	0.98
Differentiation	1 Moderate				
(reference = well)	0 Others	0.556	0.921	1.7	
	1 Poor				0.05
	0 Others	1.796	0.838	6.0	

Figure 4 Cumulative risk of first event (——), which is the sum
of local relapse (- - - -), distant metastases only (- -), and death
from other causes (- — -). Patients whose tumours were cyclin D1
negative, (n = 28) (a) or positive (n = 25) (b) are shown. There is an
increased risk of local relapse in the cyclin D1-negative group
(P = 0.01).

inhibitor of CDK2) have been reported recently and might
explain why some functional experiments have produced
paradoxical results (Peters, 1994). In particular, cyclin D1
overexpression may lead in post-mitotic neurons to apoptosis
(Freeman et al., 1994), and in human diploid fibroblasts it
might induce senescence (Dulic et al., 1993; Lucibello et al.,
1993).

Allele-specific expression analysis was carried out in the
tumours studied. In all RFLP informative cases, in which
elevated protein levels were demonstrated, an imbalance in
allele-specific relative transcript abundance was seen. Con-
versely, no tumours scoring negative for cyclin D1 expression
by immunostaining displayed an imbalance in transcript

levels (except for one tumour with a loss of heterozygosity at
the CCND1 locus). These data suggest that overexpression of
CCND1 was caused by specific genetic events affecting just
one parental allele. Thus, the elevated expression seen in the
tumour is not simply a result of non-specific up-regulation of
a gene involved in rapid cell growth but is the result of
genetic alterations directly affecting CCND1. In several cases
the transcript imbalance seen in cDNA favoured the non-
amplified allele. As the polymorphic restriction site is located
distant to the coding sequence, within the long 3'UTR, the
most likely explanation for this finding is that the mRNA
encoded by the amplified allele is preferentially spliced,
precluding amplification of the RFLP site.

The results show an association between cyclin D1
overexpression in the primary tumour and a low degree of
differentiation, little lymphocytic infiltration of the tumour,
and a low incidence of local relapse. Consistent with this
result, overexpression of other oncogene products such as the
BCL2 protein (Pezzella et al., 1993), which is known to
inhibit programmed cell death and the epidermal growth
factor receptor (EGFR) (Lee et al., 1989) that mediates the
effects of the EGF and transforming growth factor (TGF)-α
have been associated with a better patient survival. However,
because of the relatively small number of patients in this
study, the confidence interval for the local relapse hazard
ratio was wide, indicating that these data must be interpreted
with caution and require larger studies to confirm the
usefulness of cyclin D1 overexpression as a prognostic factor.

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