Histopathology 2009, **55**, 28–36. DOI: 10.1111/j.1365-2559.2009.03331.x

Expression and prognostic significance of cyclin B1 and cyclin A in non-small cell lung cancer

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Date of submission 20 July 2008
Accepted for publication 4 January 2009

Cooper W A, Kohonen-Corish M R J, McCaughan B, Kennedy C, Sutherland R L & Lee C S (2009) *Histopathology* **55**, 28–36

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Aims: Aberrant expression of cell cycle regulators has been implicated in the pathogenesis of many neoplasms, including non-small cell lung cancer (NSCLC). The aim was to examine the expression and prognostic value of cyclin B1 and cyclin A, key regulators of the G_2/M checkpoint of the cell cycle, in NSCLC and bronchial precursor lesions.

Methods and results: Immunohistochemical expression of cyclin B1 and A was examined in 90 cases of stage I–II primary NSCLC and bronchial precursor lesions using tissue microarrays. Increased cyclin B1 and A expression was found in 40.9 and 58.9% of NSCLC cases, respectively, and was significantly higher in primary NSCLC, lymph node metastases and some bronchial precursor lesions compared with normal bronchial epithelium. Increased expression of cyclin A and cyclin B1 correlated with tumour type, poorly differentiated tumours and male gender. A significant association was found between increased cyclin B1 expression and reduced survival using Kaplan–Meier survival analysis. On multivariate analysis, cyclin B1 was not an independent prognostic factor ($P = 0.067$). Cyclin A expression was not associated with survival.

Conclusions: Cyclin B1 and cyclin A are aberrantly expressed in NSCLC and some precursor lesions. Cyclin B1, but not cyclin A, shows some promise as a potential prognostic marker in NSCLC.

Keywords: cyclin A, cyclin B1, immunohistochemistry, lung neoplasm, non-small cell lung cancer, prognosis

Abbreviations: ADC, adenocarcinoma; CDK, cyclin-dependent kinase; CI, confidence interval; CIS, carcinoma *in situ;* HR, hazard ratio; LCC, large cell carcinoma; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma

Introduction

Cellular progression through the cell cycle is tightly regulated and involves two major checkpoints at the G_1/S and G_2/M transitions.[1] Cyclins are regulatory proteins of the cell cycle that are sequentially expressed and degraded at specific times during the cell cycle and combine with constitutively expressed cyclin-dependent kinases to form active catalytic units.[2] Transition through S and G_2 phase into mitosis is controlled by cyclins A and B1.[2] Cell cycle deregulation, including constitutive overexpression of cyclins, can lead to uncontrolled cell proliferation and transformation to a malignant phenotype.

Whereas small cell carcinomas of the lung uniformly follow an aggressive clinical course, non-small cell lung cancer (NSCLC) has a more variable clinical outcome. Overall survival is only about 65% in stage I NSCLC and 40% in stage II disease.[3] Determination of biological prognostic markers may help identify patients with adverse outcomes and assist in determining the most appropriate treatment protocols. A number of

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studies have investigated the prognostic significance of G_1/S transition regulators in NSCLC, particularly, cyclin D1, but the significance of cyclins involved in S phase and G_2/M transition of the cell cycle in NSCLC is not as well established.[4] Therefore in this study, we investigated the expression and subcellular localization of cyclins A and B1 in early-stage NSCLC, precursor lesions and regional metastases by examining protein expression and correlating the results with clinicopathological variables and patient survival.

Materials and methods

PATIENT COHORT

Tumour samples and clinical follow-up data were obtained from a cohort of 90 stage I–II NSCLC patients treated by surgical resection at Royal Prince Alfred Hospital, Sydney, Australia between 1997 and 1999. The cohort included 58 (64.4%) men and 32 women (35.6%) with a median age at diagnosis of 67 years (range 41–78 years) and median survival time of 68.9 months (range 1–86.7 months). Histological tumour subtypes were assessed using the World Health Organization classification.[5] Histopathological grading of tumours into well, moderate or poorly differentiated was performed in a qualitative manner based on conventional pathological criteria of architectural and cytological atypia. There were 30 (33.3%) adenocarcinomas (ADCs), 19 (21.1%) large cell carcinomas (LCCs) and 41 (45.6%) squamous cell carcinomas (SCCs). Tumours were staged using the American Joint Committee on Cancer Tumour Node Metastasis classification[6] and consisted of 72 (80%) stage I and 18 (20%) stage II tumours. The cohort included 11 patients with regional lymph node metastases. Precursor lesions were also assessed when available and there were eight cases of bronchial squamous epithelial metaplasia, two with low-grade dysplasia (mild to moderate dysplasia) and eight cases of bronchial squamous cell carcinoma *in situ* (CIS). No cases of severe dysplasia were identified. Follow-up information of ≥5 years was available for this study.

TUMOUR SAMPLES

Tissue microarrays were constructed using three to four donor cores of tumour, 1 mm in diameter, from formalin-fixed paraffin-embedded tissue blocks as previously described.[7] All tumour blocks from the resection specimens (average 12 blocks) were reviewed by a pathologist and an appropriate representative range of areas from each tumour was selected for sampling. An average of three donor cores were also taken from normal bronchial mucosa. If preneoplastic lesions were present, these were also sampled (average two cores, range 1–4). These tissue cores were arrayed into a recipient paraffin block using a tissue arraying instrument (Beecher Instruments, Silver Springs, MD, USA). Serial sections were cut from the tissue microarray blocks at 4 μm thick and mounted on glass slides.

IMMUNOHISTOCHEMISTRY

The tissue sections were deparaffinized in xylene, rehydrated through graded decreasing concentrations of alcohol, and endogenous peroxidase was blocked with 3% hydrogen peroxide/methanol for 10 min. Antigen retrieval was performed by bringing Dako Target Retrieval Solution, pH 6.0, to the boil in a pressurized antigen-decloaking chamber (Biocare Medical, Concord, CA, USA) programmed for 4 min at 124°C. After applying normal horse serum for 5 min, primary mouse antihuman monoclonal antibodies against cyclin A (Novocastra, Newcastle, UK; clone 6E6) and cyclin B1 (Novocastra; clone 7A9) were applied to the sections at concentrations of 1:100 and 1:25, respectively, for 1 h at room temperature. The antibodies were diluted in Vision Biosystem Antibody Diluent. The Vectastain Avidin–Biotin Complex kit (Vector Laboratories, Burlingame, CA, USA) was used to detect the monoclonal antibody according to the manufacturer's specifications, and the binding sites were visualized using the Diaminobenzidine kit (Dako, Carpinteria, CA, USA). The sections were counterstained with Harris's haematoxylin.

Internal controls of matched samples of normal bronchial mucosa and peripheral lung parenchyma were incorporated into the tissue arrays. Samples from normal spleen were also used in the arrays as both reference points and external controls. Negative controls were also performed by omission of the primary antibody. Whole sections of tonsil were also used as positive controls, and cyclin B1 expression (cytoplasmic in location) and cyclin A expression (nuclear in location) were seen in scattered basal and suprabasal squamous epithelial cells as well as many lymphoid cells in germinal centres and to a lesser extent in interfollicular areas.

SCORING

Immunoreactivity was scored by a specialist pathologist (W.A.C.) without knowledge of the patient's clinical details. Cytoplasmic and nuclear reactivity was

assessed and the percentage of cells with immunopositivity was estimated semiquantitatively. An average score was obtained from the multiple biopsy samples of each case. For cyclin A, high protein expression was taken as cases with ≥15% of cells with nuclear immunoreactivity.[8] For cyclin B1, high protein expression was taken as cases with ≥5% of cells with cytoplasmic immunoreractivity.[9,10]

STATISTICAL ANALYSES

Protein expression in primary carcinoma and other lesions was compared using the binomial test. Associations between protein expression and clinicopathological characteristics were compared using the Pearson χ^2 and Fisher's exact tests (two-sided). The Kaplan–Meier log rank and Cox proportional hazards regression model were used for survival analyses. The SPSS statistical software package version 13.0 was used for all analyses (SPSS Inc., Chicago, IL, USA). *P*-values of <0.05 were regarded as statistically significant.

Results

EXPRESSION OF CYCLIN A AND CYCLIN B1 IN PRIMARY NSCLC

Expression of cyclin B1 was predominantly cytoplasmic, with most cases of primary NSCLC showing scattered immunopositive tumour cells (Figure 1). The mean percentage of cells with cytoplasmic cyclin B1 expression was 7.1 ± 6.0% (mean ± SD, range 0–22.5%, median 5.3%). Increased expression of cyclin B1 was present in 36/88 (40.9%) of primary NSCLCs. Expression of cyclin A was nuclear in location and the mean percentage of cells with immunopositivity was 20.5 ± 16.6% of NSCLC cases (mean ± SD, range 0–90%, median 18.5%) (Figure 1). High expression of cyclin A was present in 53/90 (58.9%) cases.

Cyclin B1 and cyclin A expression was significantly higher in primary NSCLC than in normal bronchial epithelium ($P < 0.001$), where there was virtually no expression of either protein apart from extremely weak reactivity in a few cases (mean percentage of immunopositive cells <1%). There was no immunoreactivity for cyclin A or B1 in alveolar pneumocytes.

EXPRESSION OF CYCLIN A AND CYCLIN B1 IN NEOPLASTIC PRECURSOR LESIONS AND REGIONAL LYMPH NODE METASTASES

Cyclin B1 expression was significantly higher in squamous metaplasia ($P = 0.002$) and CIS ($P < 0.001$) compared with normal bronchus (Table 1). There was no significant difference between expression of cyclin B1 in squamous metaplasia and primary invasive carcinoma; however, expression was significantly higher in CIS than in primary invasive carcinoma ($P = 0.042$). Expression of cyclin A was significantly lower in squamous metaplasia than in invasive carcinomas ($P = 0.017$), but did not differ from normal bronchial epithelium (Table 1). Cyclin A was significantly higher in CIS than in normal bronchial epithelium ($P < 0.001$), but was not different from that in primary invasive carcinomas ($P = 0.10$). There was no significant difference between cyclin B1 or A expression in lymph node metastases compared with primary carcinomas, although expression of both proteins was significantly higher in nodal metastases compared with normal bronchus ($P < 0.001$).

CORRELATION BETWEEN EXPRESSION OF CYCLIN A AND CYCLIN B1 AND CLINICOPATHOLOGICAL VARIABLES

High cyclin B1 expression associated significantly with SCCs ($P = 0.001$) and LCCs ($P = 0.001$), whereas there was a strong inverse correlation with ADCs ($P = 0.001$) (Table 2). Increased cyclin B1 also correlated with male gender ($P = 0.027$) and poorly differentiated tumours ($P = 0.002$) and inversely with well-differentiated tumours ($P = 0.029$). Increased expression of cyclin A was significantly correlated with SCCs ($P = 0.023$), poorly differentiated tumours ($P = 0.029$) and male gender ($P = 0.014$). There was an inverse correlation with ADCs ($P = 0.002$). There was no correlation between expression of cyclin A and expression of cyclin B1 ($P = 0.09$).

RELATIONSHIP BETWEEN EXPRESSION OF CYCLIN A AND CYCLIN B1 AND PATIENT SURVIVAL

High cyclin B1 expression (≥5%) was predictive of a worse overall survival ($P = 0.016$, log rank) (Figure 2). The significant predictive value of cyclin B1 expression was maintained in stage I tumours alone ($P = 0.038$) as well as in ADCs ($P < 0.001$), but only two patients in the latter group had high cyclin B1 expression. Cox regression analysis was undertaken to determine the relative effects of cyclin B1 and other significant factors in determining any association with survival. In the entire cohort of NSCLC, when cyclin B1 and stage were included in Cox multivariate analysis, cyclin B1 was not an independent prognostic marker ($P = 0.067$). Amongst ADCs alone, significant variables in Cox univariate analysis were cyclin B1 expression

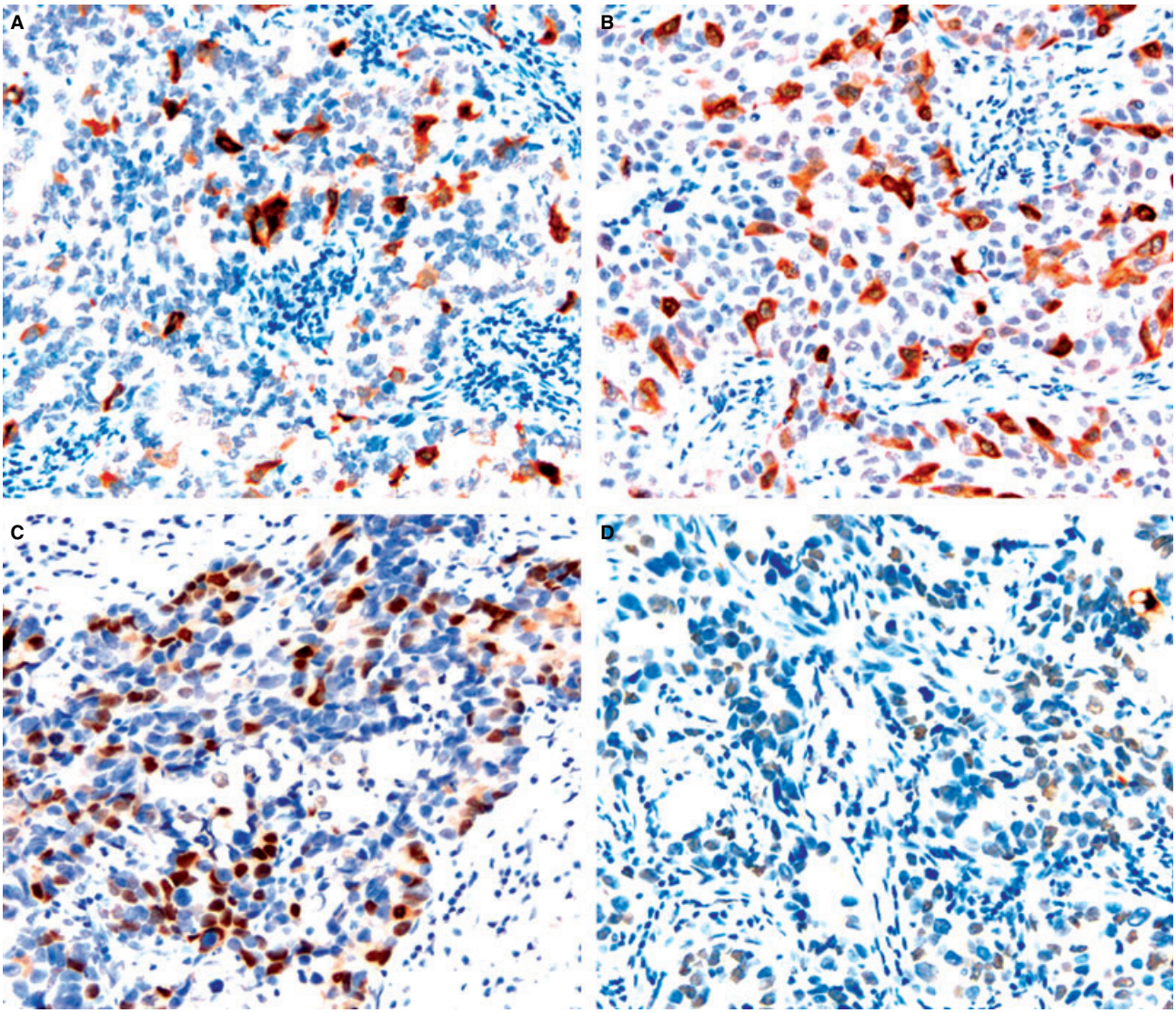

Figure 1. Immunohistochemical expression of cyclin B1 and cyclin A in non-small cell lung cancer. **A**, High cyclin B1 expression (cytoplasmic immunoreactivity) in a moderately differentiated adenocarcinoma (ADC). **B**, High cyclin B1 expression (cytoplasmic immunoreactivity) in a poorly differentiated squamous cell carcinoma. **C**, High cyclin A expression (nuclear immunoreactivity) in a poorly differentiated ADC. **D**, ADC lacking expression of cyclin B.

($P = 0.005$) and higher stage (2 versus 1) ($P = 0.045$). On multivariate analysis, both high cyclin B1 expression [$P = 0.002$, hazard ratio (HR) 20.6, 95% confidence interval (CI) 3.03, 140.23] and stage ($P = 0.018$, HR 6.08, 95% CI 1.36, 27.24) were independent prognostic markers of a worse prognosis in ADCs, but the number of cases in this tumour subgroup with high cyclin B1 expression was small.

There was no significant difference in survival of NSCLC patients based on expression of cyclin A ($P = 0.68$) (Figure 3). Survival analysis was also performed on subsets of the patient cohort based on tumour type, stage and patient gender, but no significant associations with survival were found.

Discussion

In our study of S and G_2/M phase cyclins in NSCLC, increased expression of cyclin B1 and cyclin A was found commonly in NSCLC, particularly in poorly differentiated tumours and SCCs, but less commonly in ADCs, in agreement with other studies.[11,12] High expression of cyclin B1 was seen in 40.9% of primary NSCLC cases and was significantly higher than in

Table 1. Cyclin B1 expression in non-small cell lung cancer, precursor lesions and normal bronchus

Pathology	Mean cyclin B1 expression (% positive cells)	Cases with increased cyclin B1 expression (%)	*P*-value* (compared with normal bronchus)	*P*-value* (compared with invasive cancer)	Mean cyclin A expression (% positive cells)	Cases with increased cyclin A expression (%)	*P*-value* (compared with normal bronchus)	*P* value* (compared to invasive cancer)
Normal bronchus	0.24	1.1	NA	≤0.005	0.20	0	NA	<0.001
Squamous metaplasia	2.4	28.6	≤0.002	NS	3.0	0	NS	0.017
Squamous met + LGD	0	0	NA	NA	6.75	0	†	†
CIS	11.7	100%	<0.001	0.042	17.2	66.7	<0.001	NS
Invasive carcinoma	7.1	40.9	≤0.005	NA	20.5	58.9	<0.001	NA
Metastatic carcinoma	9.6	81.8	<0.001	NS	20.4	62.5	<0.001	NS

*Binomial test.
†Too few cases to assess.
NS, not significant; NA, not applicable; LGD, low-grade dysplasia; CIS, carcinoma *in situ*.

normal bronchial epithelium. In tumours, overexpression of cyclin B1 occurs throughout the cell cycle,[13] and high expression has been reported in carcinomas of the lung,[11,12] breast,[13,14] prostate,[15] oral cavity,[16,17] larynx,[18] oesophagus,[10] stomach,[19] colon[20,21] and kidney,[22] and is typically cytoplasmic in location. In agreement with other studies,[11,12] we found increased cyclin B1 correlated with poorly differentiated tumours and SCCs as well as LCCs, whereas there was an inverse correlation with ADCs. Increased cyclin B1 expression is induced by c-Myc overexpression and loss of normal p53 function, both common occurrences in human malignancy.[13,23,24] In tumours, overexpression of cyclin B1 occurs throughout the cell cycle[13] and is predominantly cytoplasmic in location, as found in this study.[10–12,14,16,20,21,25] Cyclin B1/cyclin-dependent kinase (CDK) 2 complexes normally translocate to the nucleus upon initiation of mitosis, but these complexes remain in the cytoplasm in the setting of DNA damage[26] and there is evidence they can initiate mitosis prior to nuclear translocation.[27] Continuous overexpression or unscheduled expression of cyclin B1 either by deregulated synthesis or degradation or abnormal subcellular location may therefore be able to overcome the normal controls of the G_2/M checkpoint, leading to uncontrolled cellular proliferation.

Cyclin B1 was also significantly increased in squamous metaplasia and CIS compared with normal bronchus, although the number of cases examined was small. In other tumour types, there is also evidence of cyclin B1 overexpression occurring early in the development of malignancy, with high levels reported in precancerous dysplastic lesions of the tongue,[16] oesophagus[28] and colon.[29] Only one study has reported cyclin B1 expression in a very small sample of precancerous lung lesions consisting of a single case of dysplasia and metaplasia, and both showed increased cyclin B1.[30]

Given the role of cyclin B1 in controlling cellular progression into mitosis, it is not surprising that its up-regulation in tumours affects patient prognosis. High expression of cyclin B1 is an independent adverse prognostic factor in oesophageal SCC,[10,25,28] tongue SCC[16] and breast cancer.[9,31,32] By contrast, other studies have found no prognostic value of cyclin B1 in cancers of the colon,[20,21] stomach,[33] prostate[15] or kidney.[22] In our study of stage I–II NSCLC, high cyclin B1 was associated with poor survival on univariate analysis, but did not reach statistical significance on multivariate analysis. Others have reported that high expression of cyclin B1 is an independent adverse prognostic marker in all stage I–III NSCLC and in ADCs alone,[11] whereas another study of stage I NSCLC

Table 2. Relationship between expression of cyclins and clinicopathological characteristics

	Cyclin B1				Cyclin A			
	Low Cyclin B1 (<5%) *n*	High cyclin B1 (≥5%) *n*	χ^2, *P*	Fisher's exact, *P*	Low cyclin A (<15%) *n*	High cyclin A (≥15%) *n*	χ^2, *P*	Fisher's exact, *P*
Tumour type			<0.001*				0.004*	
ADC	27	2	<0.001*	<0.001*	20	10	0.002*	0.003*
SCC	9	31	0.001*	0.002*	12	29	0.023*	0.032*
LCC	0	19	<0.001*	<0.001*	6	13	0.29	0.43
Differentiation			0.003*				0.058	
Well	5	1	0.029*	0.040*	3	1	0.18	0.31
Mod.	22	21	0.056	0.082	24	24	0.11	0.14
Poor	9	30	0.002*	0.004*	11	27	0.029*	0.033*
Size			0.052	0.077			0.34	0.40
≤30	21	21			21	23		
>30	13	31			17	28		
Sex			0.027*	0.042*			0.014*	0.025*
Male	18	38			19	39		
Female	18	14			19	13		
Age			0.26	0.29			0.13	0.14
<67	15	28			15	29		
≥67	21	24			23	23		
Stage			0.21				0.54	
1A	13	10	0.076	0.089	12	13	0.49	0.63
1B	18	27	0.86	1.0	21	26	0.62	0.67
2A	1	4	0.33	0.65	1	4	0.30	0.39
2B	4	11	0.22	0.26	4	9	0.37	0.55
1 versus 2			0.10	0.13			0.17	0.19
BVI			0.34	0.46			0.78	1.00
Absent	34	46			35	47		
Present	2	6			3	5		
LVI			0.035*	0.077			0.45	0.69
Absent	36	46			36	47		
Present	0	6			2	5		

Table 2. *(Continued)*

	Cyclin B1				Cyclin A			
	Low Cyclin B1 (<5%) *n*	High cyclin B1 (≥5%) *n*	χ^2, *P*	Fisher's exact, *P*	Low cyclin A (<15%) *n*	High cyclin A (≥15%) *n*	χ^2, *P*	Fisher's exact, *P*
Vessel invasion			0.043*	0.065			0.26	0.38
Absent	34	41			34	42		
Present	2	11			4	10		
PNI			0.40	1.0			0.39	1.0
Absent	36	51			38	51		
Present	0	1			0	1		
Margin			†				0.12	0.23
Not involved	36	52			37	46		
Involved	0	0			1	6		
Cyclin A expression			0.09	0.12				
Low (<15%)	19	18						
High (≥15%)	17	34						

*Statistically significant ($P < 0.05$). †Too few cases to assess.
ADC, adenocarcinoma; SCC squamous cell carcinoma; LCC, large cell carcinoma; BVI, blood vessel invasion; LVI, lymphovascular invasion; PNI, perineural invasion.

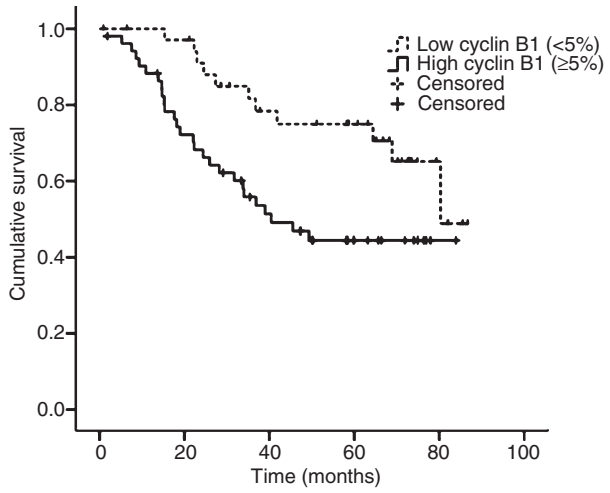

Figure 2. Probability of survival according to proportion of cells expressing cyclin B1 in all non-small cell lung cancers (Kaplan–Meier survival curve, $P = 0.016$, log rank). Low cyclin B1 expression, $n = 36$ (dotted line), high cyclin B1 expression, $n = 52$ (solid line). There were 50 censored cases.

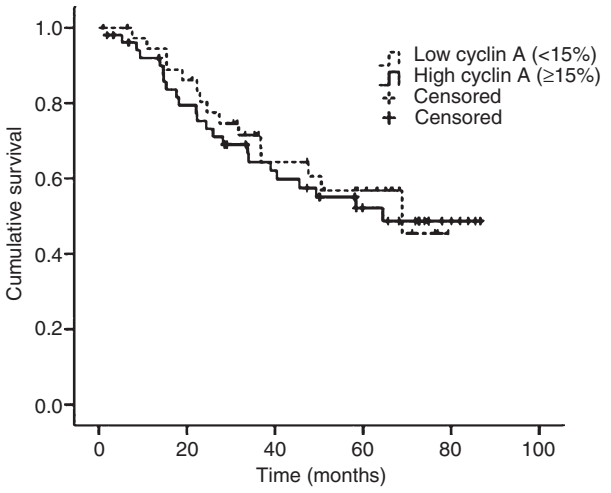

Figure 3. Probability of survival according to cyclin A expression in all non-small cell lung cancers (Kaplan–Meier survival curve, $P = 0.68$, log rank). Low cyclin A (<15%), $n = 38$ (dotted line), high cyclin A (≥15%), $n = 52$ (solid line). There were 52 censored cases.

found high cyclin B1 was associated with shorter survival in SCCs but not in ADCs or the entire group of NSCLC.[12]

We found cyclin A expression was significantly higher in NSCLC compared with normal bronchial epithelium. Others have also demonstrated cyclin A overexpression in lung cancer compared with surrounding normal

tissue using immunoblotting techniques[34] and reverse transcriptase-polymerase chain reaction.[35] Furthermore, high levels of cyclin A expression have been reported in a wide variety of carcinomas,[8,36–41] including lung cancer.[34,35] Increased cyclin A correlates with CDK2 kinase activity,[34] proportion of cells in S phase of the cell cycle[42] and proliferating cell nuclear antigen expression[35] in NSCLC, suggesting cyclin A expression reflects an active role in the cell cycle. Cyclin A expression was also increased in CIS compared with normal epithelium, whereas expression in squamous metaplasia did not differ from normal bronchial epithelium and was significantly lower than in invasive carcinoma. Similarly, elevated cyclin A expression has been described in precursor lesions of oral SCC,[8] carcinomas ex pleomorphic adenoma of the parotid gland[43] and colorectal cancer.[29] This suggests cyclin A is involved in tumorigenesis prior to development of an invasive phenotype.

There is considerable evidence that cyclin A is a predictive marker of prognosis in some human malignancies. High cyclin A has been reported as an independent adverse prognostic factor in carcinomas of the prostate,[39] breast,[38] stomach,[44] colorectum,[37] oesophagus,[36] liver[41] and kidney,[40] but a favourable prognostic factor in head and neck SCC[45] and colonic cancer.[46] The prognostic significance of cyclin A in NSCLC, however, is not clearly established. In our study, there was no significant difference in survival between patients based on levels of cyclin A expression. Similarly, in other studies of NSCLC, cyclin A was not a significant prognostic marker based on protein expression [47] or gene expression.[35] Cyclin A has been associated with poorer survival in stage I–III NSCLC,[42,48] but interestingly, and in agreement with our findings, cyclin A was not prognostically significant when only stage I–II tumours were analysed in one of these studies.[42]

In summary, we have found increased expression of cyclin A and cyclin B1 in a proportion of NSCLC. High expression of cyclin B1 was an adverse prognostic factor, whereas cyclin A had no prognostic value. Cyclin B1 shows promise as a useful clinical marker of prognosis and could potentially assist in determining appropriate management of patients.

Competing interests

None to declare.

Acknowledgements

M.R.J.K-C is a Cancer Institute NSW Fellow. The authors thank the Cancer Institute NSW, National Health and Medical Research Council and the RT Hall Trust for their support.

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