ORIGINAL ARTICLE

Inverse correlation between high level expression of cyclin E and proliferation index in transitional cell carcinoma of the bladder

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Background/Aims: Overexpression of the G1 cyclins, D1 and E, and/or downregulation of $p27^{Kip1}$ allow uncontrolled tumour cell proliferation. This study investigated the relation between these three cell cycle proteins and tumour proliferation in bladder cancer.
Method: Nuclear expression of cyclin D1, cyclin E, and $p27^{Kip1}$ was determined immunohistochemically in 52 primary transitional cell carcinomas, and the Ki-67 proliferation marker was also assessed. For each protein, the percentage of positive tumour cell nuclei was determined and analysed as a continuous variable.
Results: Advancing tumour grade and pathological stage were accompanied by increasing proliferation indices, but decreasing $p27^{Kip1}$ and cyclin D1 expression, with no significant change in cyclin E expression. Overall, cyclin D1 and E expression did not correlate with proliferation. However, in cyclin D1 overexpressing tumours ($\geqslant$ 5% nuclei positive), the level of cyclin D1 expression positively correlated with proliferation. The correlation between cyclin E expression and proliferation changed from positive to negative with increasing levels of cyclin E expression, accompanied by a coordinate increase in $p27^{Kip1}$ expression. Overall, there was an inverse association between $p27^{Kip1}$ expression and proliferation. However, a subset of tumours displayed high proliferation indices despite high $p27^{Kip1}$ expression. The G1 cyclin index (sum of the level of expression of cyclins D1 and E) correlated positively with proliferation in superficial but not muscle invasive tumours. This correlation was stronger when the G1 cyclin index was adjusted for $p27^{Kip1}$ expression.
Conclusion: These findings support a role for these proteins in the proliferation, differentiation, and progression of bladder transitional cell carcinomas.

Multiple positive and negative regulators control progression through the cell cycle. The cyclins are a group of positive regulatory proteins, which in association with their catalytic subunits, the cyclin dependent kinases (CDKs), mediate important cell cycle transitions.[1–3] The activity of the cyclin–CDK complexes is negatively regulated by the CDK inhibitors.[1–3] At the G1–S boundary, these proteins control entry of the cell into its DNA replicative S phase. After passing through the restriction point in late G1, cells are generally committed to completing at least one round of the cell cycle. At this point, the G1–S transition is tightly regulated to ensure that damaged DNA is not replicated. Altered regulation of this crucial checkpoint can result in uncontrolled cell proliferation and the transmission of damaged DNA to daughter cells.[2] Not surprisingly, regulatory defects involving the G1–S checkpoint have been observed in many human cancers.[1–3]

> ''The cyclin dependent kinase (CDK) inhibitor, $p27^{Kip1}$ blocks the activity of cyclin D1–CDK4/6 and cyclin E–CDK2, and is an important negative regulator of the G1–S transition''

Cyclins D1 and E are important positive regulators of the G1–S transition. Cyclin D1 is expressed in early G1 in response to mitogenic signals and is primarily involved in G1 phase progression.[3] Its expression is maximal in mid-G1 phase. Cyclin E is expressed in late G1 and facilitates S phase entry.[3] Both G1 cyclins form active complexes with their respective CDKs (cyclin D1, CDK4/6; cyclin E, CDK2) to phosphorylate three pocket proteins of the retinoblastoma family, pRB, p107, and p130, which then release the transcription factors required for S phase entry.[4] In vitro, forced overexpression of cyclin D1 and cyclin E accelerates the G1–S transition, resulting in a shortened G1 phase and reduced growth factor requirement.[5] Although both cyclin D1 and E have a rate limiting role in G1–S progression, only cyclin E has been shown to be essential for this process.[6 7] Furthermore, cyclin D1 is dependent on wild-type pRb for its role in cell cycle progression,[8 9] whereas cyclin E can function in RB negative cells.[6 10] Forced overexpression of G1 cyclins can overcome control of the G1–S checkpoint, leading to unrestrained proliferation, so that these proteins are thought to have oncogenic potential. Overexpression of cyclins D1 and E, as a result of gene amplification or altered post-transcriptional regulation, has been observed in many human cancers, and generally correlates with advancing degree of malignancy and poor prognosis.[1–3 11 12]

In contrast, the CDK inhibitor, $p27^{Kip1}$ blocks the activity of cyclin D1–CDK4/6 and cyclin E–CDK2, and is an important negative regulator of the G1–S transition.[1–3] The concentration of $p27^{Kip1}$ is normally high in resting cells, but declines in response to proliferative stimuli.[13] Forced overexpression of $p27^{Kip1}$ in cell lines leads to G1 arrest.[14] Furthermore, antisense inhibition of $p27^{Kip1}$ protein synthesis in proliferating

Abbreviations: CDK, cyclin dependent kinase; PBS, phosphate buffered saline; pRb, retinoblastoma protein; TCC, transitional cell carcinoma of the bladder

cells blocks re-entry into the resting or G0 phase of the cell cycle.[15] As a negative regulator of cell cycle progression, $p27^{Kip1}$ is also considered to be a tumour suppressor.[1] Progressive downregulation of $p27^{Kip1}$ is a common finding in different types of cancer, and is associated with advanced disease and reduced survival.[1–3 16] Post-translational mechanisms, mainly increased ubiquitin mediated proteolysis, account for reduced concentrations of $p27^{Kip1}$, because it is rarely mutated in human cancers.[1 16]

In transitional cell carcinoma of the bladder (TCC), cyclin D1 overexpression correlates with low tumour grade and stage, and has been shown to be a predictor of early recurrence.[17–20] Loss of $p27^{Kip1}$ expression in superficial TCCs has been correlated with tumour grade and reduced survival.[21] More recently, it has been reported that reduced expression of both cyclin E and $p27^{Kip1}$ predicts poor survival in TCC.[22] However, these three cell cycle regulators have not been studied concurrently in bladder cancer and their association with the proliferation index has not been explored.

In our present study, we determined the nuclear expression of cyclin D1, cyclin E, and $p27^{Kip1}$ in a series of 52 primary TCCs. The results were correlated with tumour grade and pathological stage. In addition, we studied the relation between the expression of these proteins and the proliferation index, as assessed by Ki-67 expression. We observed a paradoxical, negative correlation between the level of cyclin E expression and the proliferation index in tumours that displayed a high level of cyclin E expression ($\geqslant$ 30% nuclei positive). In contrast, the level of cyclin E expression was positively correlated with the proliferation index in low expressors of cyclin E ($<$ 30%). We propose an explanation for these findings and discuss their implications.

MATERIALS AND METHODS

Patients and tissue samples

Formalin fixed, paraffin wax embedded tissue blocks from 52 patients with a confirmed diagnosis of primary TCC of the bladder were obtained from the department of histopathology and the human biomaterials resource centre of the Imperial College School of Medicine, Hammersmith Hospital Campus, London, UK. The median age of the patients was 71 years (range, 35–95). Forty six were men and six were women. Tumour specimens were obtained from 34 transurethral resections, six cystectomies, and 12 biopsies (transurethral resection or cold cup). In all cases, only samples in which the presence of detrusor muscle was identified histologically were selected to ensure accuracy in reflecting pT category. None of the patients had received irradiation or chemotherapy. Seven tumours were classified according to the World Health Organisation[23] as well differentiated (grade 1), 26 as moderately differentiated (grade 2), and 19 as poorly differentiated (grade 3). In accordance with the classification of the American Joint Committee on Cancer,[24] there were 14 pTa, 19 pT1, seven pT2, and 12 pT3/4 tumours. In 29 tumours, a papillary phenotype was identified.

Immunohistochemistry

Well characterised, mouse monoclonal antibodies against Ki-67 antigen (MIB-1; Immunotech, Marseille, France), $p27^{Kip1}$ (Transduction Laboratories, Lexington, Kentucky, USA), and cyclin E (HE12; Santa Cruz Biotechnology, Santa Cruz, California, USA), each at a 1/400 dilution, and cyclin D1 (P2D11F11; Novocastra Laboratories, Newcastle upon Tyne, UK), at a 1/20 dilution, were used. Serial sections (5 µm thick) were mounted on poly-L-lysine coated slides. Sections were dewaxed, rehydrated, and washed in phosphate buffered saline (PBS; pH 7.2). To enhance antigen exposure, slides were immersed in prewarmed 0.01M citrate buffer (pH 6.0) and microwaved at 750 W (Philips M902), three times for five minutes each. Sections were then allowed to cool for 20 minutes. Endogenous peroxidase activity was blocked with 0.3% vol/vol H_2O_2 for 30 minutes. Sections were washed in PBS and incubated with 10% normal rabbit serum (Dako, Glostrup, Denmark) for 30 minutes to block non-specific binding sites. This was followed by overnight incubation with each primary antibody at 4°C. Secondary antibody (biotinylated rabbit antimouse immunoglobulin; 1/200 dilution; Dako) was applied for 45 minutes at room temperature. After sections were washed in PBS, avidin–biotin–horseradish peroxidase complexes (1/100 dilution; Dako) were applied for 30 minutes. 3,3′-Diaminobenzidine solution (0.05% wt/vol; Sigma, Poole, Dorset, UK) was used as the final chromogen and sections were counterstained with Cole's haematoxylin (Pioneer Research Chemicals, Essex, UK). Sections in which the primary antibody was replaced by PBS were used as negative controls. Sections with stromal lymphoid follicles clearly displaying an inverse relation between $p27^{Kip1}$ and Ki-67 expression in lymphocytes were included as positive controls for $p27^{Kip1}$ and Ki-67. In addition, stromal or peritumoural lymphocytes were present in most cases and served as internal controls for $p27^{Kip1}$ and Ki-67. As previously reported by Dutta *et al*,[25] we consistently found endothelial cytoplasmic crossreactivity with the HE12 anti-cyclin E antibody used. Whereas they had suggested that this property of the HE12 antibody could be used to assess tumour vasculature, we found it to be a consistent and useful positive internal control for cyclin E immunoreactivity. A breast tumour specimen with known cyclin D1 and E expression was used as a positive control for cyclins D1 and E.

Evaluation of immunostaining

Slides were reviewed independently by three investigators, without knowledge of the clinical data (AAK, KSC, ENL). Interobserver discrepancies were resolved using a double headed microscope. Only nuclear expression was recorded. The number of distinctly positive tumour cell nuclei was counted under high power (×400) using a 10 × 10 eyepiece grid. In total, 1000 tumour cells were assessed. The number of positive nuclei was expressed as a percentage of all tumour cell nuclei counted. For Ki-67, $p27^{Kip1}$, and cyclin E, a single case for each antibody was excluded from the final analysis because of inadequate staining.

Statistical analysis

Individual staining indices (percentage of nuclei positive) were analysed as continuous variables. Cut off points for high versus low expression, based on median levels of expression, were included when appropriate. The Mann-Whitney U and Kruskall-Wallis tests were used for comparing two and three different groups, respectively. Correlations between variables were assessed by Spearman's rank correlation test (r_s = correlation coefficient). Multiple logistic regression was used to assess which independent variables were most strongly associated with a dichotomised dependent variable. All p values were two tailed and considered significant when $\leqslant$ 0.05. Statistical analyses were performed on Stata 6.0 (Stata Corporation, College Station, Texas, USA).

RESULTS

Expression of cyclin D1, cyclin E, $p27^{Kip1}$, and Ki-67

Figures 1 and 2 show typical examples of the immunohistochemical expression of cyclin D1, cyclin E, $p27^{Kip1}$, and Ki-67 in representative TCCs. The criteria for scoring were based on previous experience from our group, in addition to review of literature. Nuclear expression of cyclin D1 ranged from 0% to 82.6% of tumour cells. Less than 5%, 5–30%, and $>$ 30%

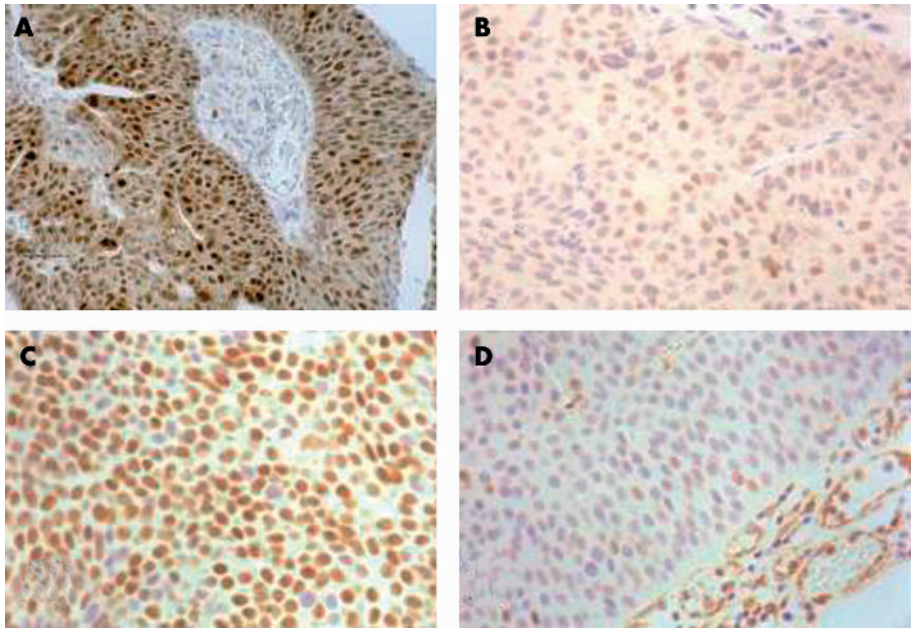

Figure 1 Immunohistochemical staining of representative bladder transitional cell carcinomas with mouse monoclonal antibodies against cyclin D1 and cyclin E (HE12). (A) Strong nuclear and cytoplasmic expression of cyclin D1 in a grade 2, superficial tumour (original magnification, ×200). (B) Low nuclear expression of cyclin D1 in a grade 3, muscle invasive tumour. (C) Strong nuclear expression of cyclin E in a grade 2, superficial tumour. (D) Low nuclear expression of cyclin E in a grade 2, muscle invasive tumour. Note the endothelial cytoplasmic crossreactivity with the HE12 anti-cyclin E antibody used (B–D: original magnification, ×400).

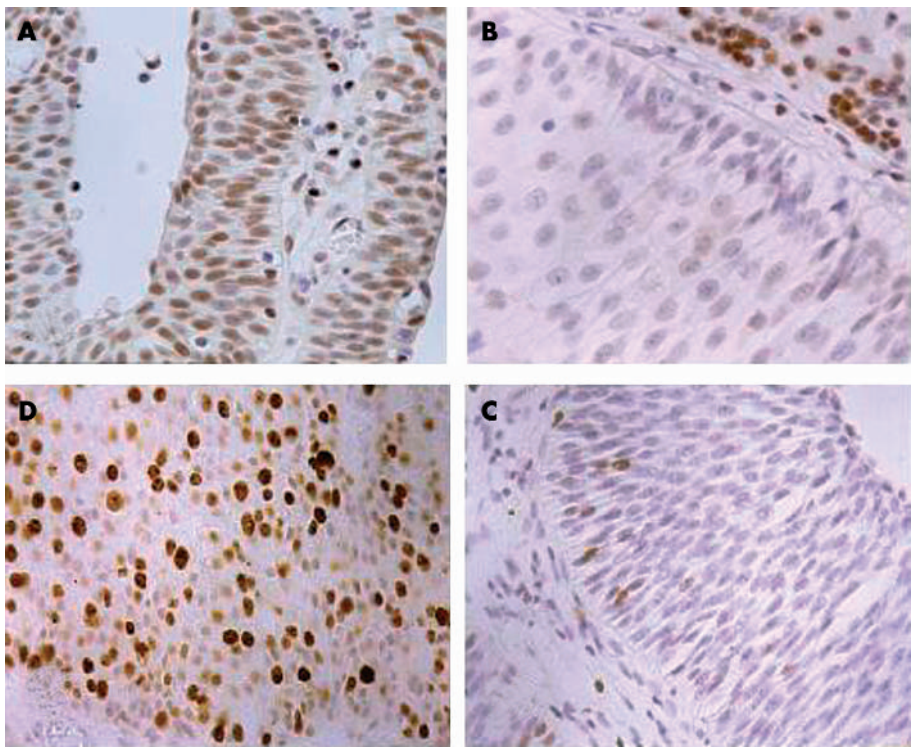

Figure 2 Immunohistochemical staining of representative bladder transitional cell carcinomas with mouse monoclonal antibodies against $p27^{Kip1}$ and Ki-67 (Mib1). (A) Strong nuclear expression of $p27^{Kip1}$ in a grade 1, papillary tumour. (B) No nuclear expression of $p27^{Kip1}$ in a grade 2, muscle invasive tumour, showing strong staining of stromal lymphocytes. (C) Strong nuclear expression of Ki-67 in a grade 3, muscle invasive tumour. (D) Low nuclear expression of Ki-67 in a grade 1, papillary tumour (A–D: original magnification, ×400).

nuclear expression of cyclin D1 was seen in 31, 13, and eight of the 52 cases, respectively. Six cases had > 50% expression.

Cyclin E expression ranged from 0% to 78.1% in the tumour cells. Less than 5%, 5–30%, and > 30% cyclin E expression was seen in 10, 16, and 25 of 51 tumours, respectively. In 14 tumours > 50% of nuclei were positive.

$p27^{Kip1}$ nuclear reactivity ranged from 0% to 67.4% in the tumour cells. Less than 30%, 30–50%, and > 50% $p27^{Kip1}$ expression was found in 29, nine, and 13 of 51 cases, respectively.

Ki-67 protein expression ranged from 0.1% to 99.0% in the tumour cells. Less than 20%, 20–50%, and > 50% tumour cell nuclei were Ki-67 positive in 29, 17, and five of 51 cases, respectively.

Combined alterations in cell cycle protein expression

Low expression of $p27^{Kip1}$ (< 30% nuclei positive) was seen in 29 of 51 cases, whereas cyclins D1 and E were overexpressed (≥ 5% and ≥ 30% nuclei positive, respectively) in 21 of 52 and 25 of 51 of all cases, respectively. Overexpression of one or both cyclins was seen in 33 of 50 tumours. Cyclins D1 and E were co-overexpressed in 11 cases. In 22 tumours, a single G1 cyclin was

overexpressed: cyclin D1 in seven and cyclin E in 15. The most frequent combination was that of low cyclin D1, low cyclin E, and low $p27^{Kip1}$ expression, which was seen in 12 of 50 cases. The combination closest to a normal profile—low cyclin D1 and E expression but high $p27^{Kip1}$ expression—was present in only four of 50 cases. Five of 50 cases displayed overexpression of both cyclins D1 and E, in addition to downregulation of $p27^{Kip1}$.

Correlation between expression of cell cycle proteins, proliferation index, and histopathological variables

The proliferation index (as determined by Ki-67 expression) increased with increasing tumour grade ($p = 0.012$) and pathological stage, and was significantly higher in muscle invasive ($\geqslant$ pT2) and non-papillary tumours than in their superficial and papillary counterparts ($p = 0.002$ and 0.03, respectively; table 1). In contrast, $p27^{Kip1}$ and cyclin D1 expression decreased with increasing grade and depth of tumour invasion, and were significantly higher in superficial tumours than in muscle invasive ones ($p = 0.047$ and 0.008, respectively). These correlations were notably more significant for cyclin D1 than for $p27^{Kip1}$. Cyclin D1 was also strongly associated with papillary morphology ($p < 0.0001$), whereas $p27^{Kip1}$ was not ($p = 0.69$). Although a modest decrease in the level of expression of cyclin E was seen with increasing grade and depth of tumour invasion, these differences were not significant. There was also no association between cyclin E expression and papillary morphology ($p = 0.96$). We also analysed levels of cyclin D1, cyclin E, $p27^{Kip1}$, and Ki-67 expression together as continuous variables using multiple logistic regression, with muscle invasion (presence versus absence) as a dichotomised outcome variable. Only proliferation index (Ki-67 expression) and cyclin D1 expression were found to be significantly associated with the presence or absence of muscle invasion in this analysis ($p = 0.015$ and 0.045, respectively).

Correlation between expression of cell cycle proteins and proliferation index

Overall, the level of expression of cyclins D1 and E did not correlate with the proliferation index ($r_s = -0.04$, $p = 0.8$, fig 3A; and $r_s = 0.21$, $p = 0.15$, fig 3C; respectively). However, when low expressors of cyclin D1 ($< 5\%$ nuclei positive, $n = 31$) were excluded, we saw a significant positive correlation between cyclin D1 expression and proliferation index ($r_s = 0.58$, $p = 0.008$, $n = 20$; fig 3B).

We next evaluated the relation between cyclin E expression and the proliferation index in high expressors of cyclin E ($\geqslant 30\%$ nuclei positive, $n = 25$) versus low expressors ($< 30\%$ nuclei positive, $n = 25$). Paradoxically, high expressors of cyclin E displayed a significant negative correlation between the level of cyclin E expression and the proliferation index ($r_s = -0.68$, $p = 0.002$; fig 3D). In contrast, this association was reversed in low expressors of cyclin E ($r_s = 0.56$, $p = 0.004$; fig 3E). To determine the level of cyclin E expression at which this correlation changed from positive to negative, the slope of the regression line for each successive 20% increase in the level of cyclin E expression is shown (fig 3F). The switch from a positive to a negative association occurred at $> 40\%$ expression of cyclin E.

To explain these opposing relations, we examined the expression of $p27^{Kip1}$, a major inhibitor of cyclin E associated kinase activity. We found that high expressors of cyclin E ($\geqslant 30\%$) compared with low expressors had significantly higher levels of $p27^{Kip1}$ expression (mean, 35.0%; SEM, 4.5% versus mean, 20.6%; SEM, 4.0%; $p = 0.025$). Furthermore, although there was an overall positive correlation between $p27^{Kip1}$ and cyclin E expression ($r_s = 0.32$, $p = 0.02$; fig 4A), we observed a difference in this association between high and low expressors of cyclin E. High expressors of cyclin E

Table 1 Nuclear expression of Ki-67, $p27^{Kip1}$, cyclin D1, and cyclin E in transitional cell carcinoma of the bladder in relation to patient and histopathological variables

	Ki-67	p27Kip1	Cyclin D1	Cyclin E
All patients	24.3 (3.2; 51)	27.7 (3.1; 51)	14.9 (3.2; 52)	31.4 (3.3; 51)
Age (years)				
<65	38.1 (8.9; 14)	19.0 (6.3; 14)	7.7 (5.1; 14)	23.4 (5.5; 14)
⩾65	19.0 (2.3; 37)	31.1 (3.5; 37)	17.6 (3.9; 38)	34.4 (4.0; 37)
p Value	0.12	0.09	0.06	0.15
Sex				
Male	23.4 (3.4; 45)	28.0 (3.4; 45)	16.3 (3.6; 46)	32.6 (3.6; 45)
Female	30.5 (7.8; 6)	25.9 (6.6; 6)	4.7 (4.7; 6)	21.9 (7.2; 6)
p Value	0.18	0.88	0.18	0.41
Papillary morphology				
Present	16.6 (2.0; 28)	28.5 (4.0; 28)	26.3 (4.8; 29)	31.7 (4.6; 28)
Absent	33.6 (6.0; 23)	26.8 (5.0; 23)	0.6 (0.4; 23)	31.0 (4.9; 23)
p Value	0.031	0.69	<0.0001	0.962
Tumour grade				
G1	12.5 (3.9; 7)	45.7 (7.9; 6)	32.2 (12.7; 7)	45.2 (11.7; 7)
G2	17.2 (2.2; 25)	27.8 (4.1; 26)	18.9 (4.6; 26)	26.4 (4.3; 25)
G3	37.8 (6.8; 19)	22.1 (5.2; 19)	3.1 (2.5; 19)	32.9 (5.1; 19)
p Value	0.012*	0.08*	0.006*	0.207*
Pathological stage				
pTa	13.5 (2.7; 14)	34.2 (6.6; 14)	25.2 (8.1; 14)	32.9 (6.7; 14)
pT1	17.5 (2.7; 18)	30.4 (4.8; 18)	16.2 (4.9; 19)	34.5 (6.7; 18)
pT2	34.9 (11.0; 7)	30.7 (9.5; 7)	14.3 (9.3; 7)	25.3 (6.5; 7)
pT3/4	40.6 (8.8; 12)	14.6 (4.6; 12)	1.3 (1.1; 12)	28.6 (5.1; 12)
p Value				
Invasive (⩾pT1)	28.3 (4.0; 37)	25.3 (3.4; 37)	11.1 (3.1; 38)	30.8 (3.8; 37)
p Value	0.03†	0.17†	0.09†	0.87†
Superficial (⩽pT1)	15.8 (1.9; 32)	32.1 (3.9; 32)	20.0 (4.4; 33)	33.8 (4.7; 32)
Muscle invasive (⩾pT2)	38.5 (6.7; 19)	20.5 (4.8; 19)	6.1 (3.6; 19)	27.4 (3.9; 19)
p Value	0.002	0.047	0.008	0.54

Values are mean percentage of nuclei positive (SEM and number of cases in parentheses).
*By Kuskall-Wallis test; all other p values by Mann-Whitney U test.
†Compared with pTa (non-invasive subgroup).

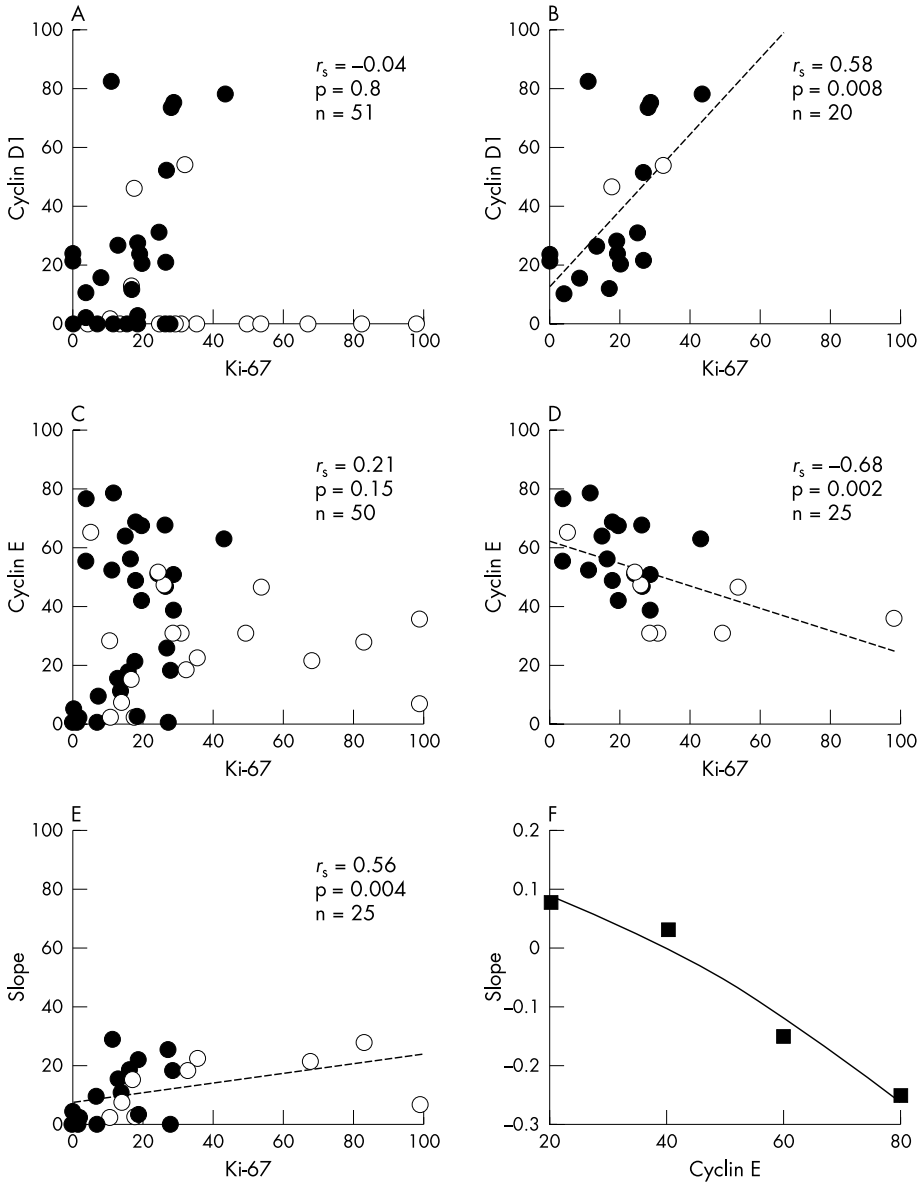

Figure 3 Scatter diagrams of cyclin D1 or cyclin E versus Ki-67 expression (percentage of nuclei positive). (A) All cases together, showing no correlation between cyclin D1 and Ki-67 expression; (B) high expressors of cyclin D1 (⩾ 5% nuclei positive), showing a positive correlation; (C) all cases together, showing no correlation between cyclin E and Ki-67 expression; (D) high expressors of cyclin E (⩾ 30% nuclei positive), showing a negative correlation; and (E) low expressors of cyclin E (< 30% nuclei positive), showing a positive correlation (r_s, Spearman's rank correlation coefficient). Filled and unfilled circles represent superficial and muscle invasive tumours, respectively. (F) shows the slope of the regression line for each successive 20% increase in cyclin E expression. The relation between cyclin E expression and the proliferation index changed from positive to negative at > 40% cyclin E expression.

displayed a positive correlation between $p27^{Kip1}$ and cyclin E expression, approaching significance (r_s = 0.38, p = 0.06), whereas in low expressors of cyclin E, there was no significant correlation (r_s = −0.24, p = 0.24). More importantly, as shown in fig 4B, there was a pronounced increase in the slope of the regression line coincident with the > 40% level of cyclin E expression.

Because cyclin D1 is known to sequester $p27^{Kip1}$, thereby decreasing the pool of $p27^{Kip1}$ available for inhibition of cyclin E–CDK2 activity, it was also important to determine the level of expression of cyclin D1. However, we did not find a significant difference in its level of expression in high versus low expressors of cyclin E (mean, 18.4%; SEM, 5.6% versus mean, 11.5%; SEM, 3.4%, respectively; p = 0.599).

An overall inverse correlation between $p27^{Kip1}$ expression and proliferation index was found (r_s = −0.36, p = 0.01; fig 4C). However, eight tumours displayed high proliferation indices (⩾ 20%), despite high level expression of $p27^{Kip1}$ (⩾ 30%). Each of these showed overexpression of one or both cyclins (D1 and/or E). Four of these were poorly differentiated and muscle invasive tumours.

When superficial TCCs were compared with muscle invasive tumours, we found weak positive correlations between the proliferation index and expression levels of both cyclins D1 and E in superficial but not in muscle invasive tumours (r_s = 0.37, p = 0.04 and r_s = 0.33, p = 0.07 versus r_s = −0.29, p = 0.22 and r_s = 0.04, p = 0.87, respectively). Although there was no significant correlation between the level of $p27^{Kip1}$ expression and the proliferation index among superficial tumours (r_s = −0.29, p = 0.108), a corresponding negative correlation was seen in the muscle invasive group (r_s = −0.45, p = 0.05).

To assess whether there were any cooperative effects of the expression of both G1 cyclins on tumour proliferation, we determined the sum of their levels of expression as a combined index of G1 cyclin expression. Although overall this index did not correlate with the proliferation index (r_s = 0.21, p = 0.145, n = 50; fig 5A), there was a significant positive correlation between the proliferation index and the combined levels of expression of cyclins D1 and E in superficial TCCs but not in muscle invasive tumours (r_s = 0.48, p = 0.008 versus r_s = −0.15, p = 0.534;

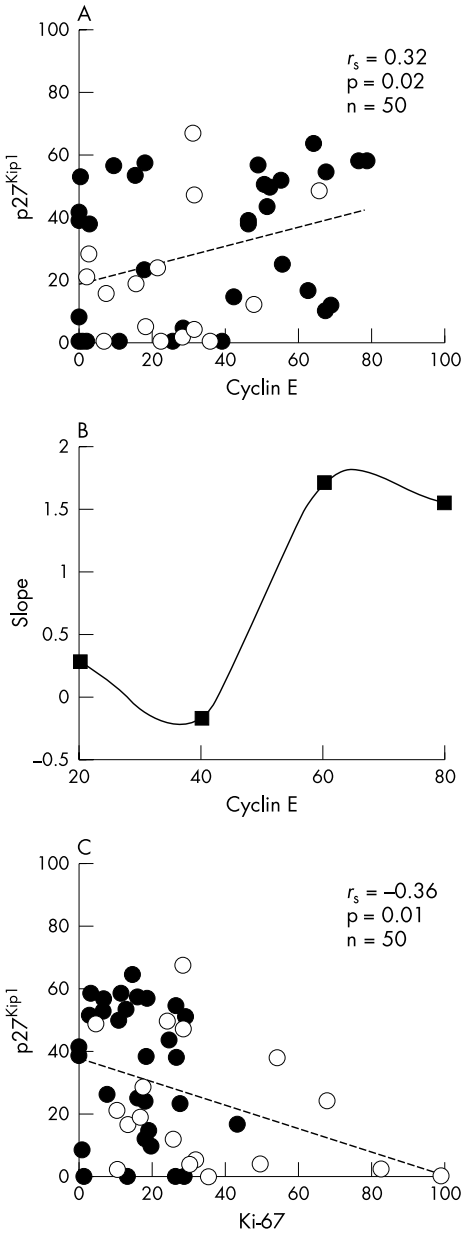

Figure 4 (A) Scatter diagram of $p27^{Kip1}$ versus cyclin E expression (percentage of nuclei positive), showing a weak correlation between $p27^{Kip1}$ and cyclin E expression. (B) The slope of the regression line for each successive 20% increase in cyclin E expression, showing a pronounced increase in the correlation between $p27^{Kip1}$ and cyclin E expression, coincident with a level of > 40% cyclin E expression. (C) Scatter diagram of $p27^{Kip1}$ versus Ki-67 expression (percentage of nuclei positive), showing a weak negative correlation between $p27^{Kip1}$ and Ki-67 expression (r_s, Spearman's rank correlation coefficient). Filled and unfilled circles represent superficial and muscle invasive tumours, respectively.

fig 5B). To account for the inhibitory effect of $p27^{Kip1}$ on G1 cyclin activity, we simply subtracted the level of $p27^{Kip1}$ expression from the G1 cyclin index for each tumour, yielding a composite expression index of all three cell cycle proteins. This index was significantly correlated with the proliferation index for all TCCs together (r_s = 0.40, p = 0.005, n = 49; fig 5C), and even more strongly so in the superficial group of TCCs (r_s = 0.59, p = 0.001, n = 30; fig 5D), but not in muscle invasive tumours (r_s = 0.23, p = 0.324).

There was no correlation between $p27^{Kip1}$ and cyclin D1 expression (r_s = 0.02, p = 0.90), or between cyclin D1 and E expression (r_s = 0.14, p = 0.35). When levels of cyclin D1, cyclin E, and $p27^{Kip1}$ expression were analysed together as continuous variables using multiple logistic regression, with the proliferation index included as a dichotomised dependent variable (high versus low, at a 20% cut off), we found that only $p27^{Kip1}$ expression was significantly associated with the proliferation index (p = 0.045).

DISCUSSION

Overexpression of the G1 cyclins, D1 and E, and/or downregulation of $p27^{Kip1}$ are potent mechanisms that allow tumour cells to undergo uncontrolled proliferation.[2–4] In normal tissues, concentrations of $p27^{Kip1}$ and G1 cyclins are closely related to cell proliferation. However, in tumours the expression is frequently deregulated relative to tumour proliferation status.[25 26] There is also inappropriate expression relative to cell cycle phase, often leading to a disturbed order of expression.[26]

In our immunohistochemical study, we determined the nuclear expression of three key regulators of the G1–S transition, cyclin D1, cyclin E, and $p27^{Kip1}$, in primary TCC. The expression of these cell cycle proteins was correlated with grade, pathological stage, and the proliferation index. The inclusion of biopsied samples limited accurate classification of the pathological stage, but there was adequate tissue in all cases to classify tumour grade.

The expression of cyclin D1, cyclin E, and $p27^{Kip1}$ in our study was comparable to that reported previously, including the results from studies on animal models of TCC.[17–22 27 28] Advancing tumour grade and depth of invasion were accompanied by increasing proliferation indices but decreasing cyclin D1 and $p27^{Kip1}$ expression, whereas cyclin E expression did not change significantly, but there are conflicting reports of cyclin D1 expression in TCC. Three studies found an association between cyclin D1 overexpression and low tumour grade and/or stage,[17 18 20] but others have reported no relation in TCC.[19] These differences may result from the different antibodies, methods, and/or cut off points used. Unlike previous reports, we decided to analyse levels of cyclin D1 expression as a continuous variable. We also found a strong association between cyclin D1 expression and papillary phenotype, as reported by others.[17 20] Our results suggest that cyclin D1 overexpression may be an important early event in the progression of TCCs. In addition, its association with well differentiated, papillary tumours suggests that cyclin D1 may also play a role in tumour differentiation.

> "Our results suggest that cyclin D1 overexpression may be an important early event in the progression of transitional cell carcinomas"

However, it is not clear whether the apparent downregulation of cyclin D1 with increasing tumour grade and stage occurs in the same TCC cells, or is the result of a clonal selection process, whereby the accumulation of other mutations (for example, RB and TP53)[29] possibly confers a greater growth advantage to cyclin D1 negative cells. The role of cyclin D1 as a positive regulator of cell cycle progression is dependent on the presence of wild-type pRb.[6–8] Moreover, pRb can directly induce cyclin D1 gene expression by interacting with the D1 promoter.[30] Cyclin D1 overexpression and inactivating RB mutations have not been found concurrently in tumour cells,[8 31–33] and are considered mutually exclusive events in TCC.[18] It is likely that the apparent downregulation of cyclin D1 is a manifestation of the clonal selection hypothesis outlined above, as opposed to a decrease in cyclin D1 expression per se. Further studies are

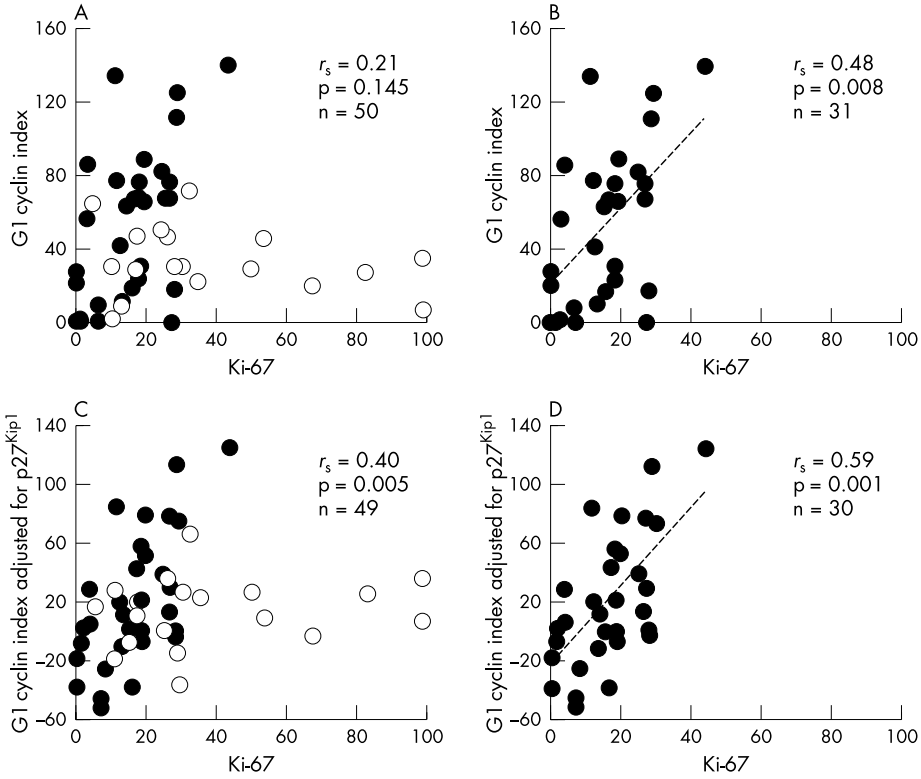

Figure 5 Scatter diagrams of the G1 cyclin index (sum of the levels of expression of cyclins D1 and E) versus Ki-67 expression (percentage of nuclei positive). (A) All cases together, showing no correlation between G1 cyclin index and Ki-67 proliferation index. (B) Superficial tumours only, showing a positive association. (C) All cases together, with the G1 cyclin index adjusted for $p27^{Kip1}$ expression (level of $p27^{Kip1}$ expression subtracted from G1 cyclin index), showing a positive correlation. (D) Superficial tumours only, showing an even stronger positive correlation (r_s, Spearman's rank correlation coefficient). Filled and unfilled circles represent superficial and muscle invasive tumours, respectively.

needed to verify this hypothesis in bladder cancer, although our data are consistent with the hypothesis that a cyclin D1 dependent pathway determines the evolution of a group of well differentiated low-stage papillary TCCs, whereas tumours that evolve via cyclin D1 independent mechanisms are less differentiated and pathologically more aggressive.

We examined the relation between the level of G1 cyclin expression and the proliferation index in superficial versus muscle invasive TCCs. Positive correlations were found between the levels of cyclin D1 and E expression and the proliferation index in superficial TCCs, but not in muscle invasive tumours. The ectopic expression of both cyclins D1 and E has been shown to have an additive effect on G1 phase progression.[7] To assess whether there were any cooperative effects of the expression of both G1 cyclins on proliferation, we assessed a combined index of G1 cyclins, defined as the sum of their individual levels of expression. There was a significant positive correlation between the proliferation index and this combined index of G1 cyclin expression in superficial but not muscle invasive TCCs. The G1 cyclin index adjusted for $p27^{Kip1}$ expression was positively correlated with the proliferation index in all TCCs, but most strongly in the superficial group. Tumour proliferation in early stage TCC may be predominantly driven by the G1 cyclins, whereas in more advanced tumours, other proliferative mechanisms are involved. These observations are consistent with the reported accumulation of RB and TP53 mutations in advanced TCCs.[29 34 35] Moreover, Wright *et al* have reported that the Ki-67 proliferation indices for pRb negative and p53 positive TCCs are nearly twice those for pRb positive and p53 negative tumours.[36]

A positive relation between cyclin E expression and the proliferation index has been reported in several different tumour types.[37–40] Our study found a negative correlation between high levels of cyclin E expression and the proliferation index. This correlation changed from positive to negative with increasing levels of cyclin E expression. Kamai *et al* suggested that cyclin E overexpression might be characteristic of a subset of bladder cancer, especially the early stages of invasion.[40a]

$p27^{Kip1}$ is a major negative regulator of cyclin E and can render it functionally inactive.[1–3] High expressors versus low expressors of cyclin E had significantly higher levels of $p27^{Kip1}$ expression, and overall there was a positive correlation between $p27^{Kip1}$ and cyclin E expression.[23 41] Cyclin D1 can effectively sequester $p27^{Kip1}$ away from cyclin E–CDK2 complexes,[3 42] so that changes in the level of cyclin D1 can result in altered cyclin E associated kinase activity, without a corresponding change in $p27^{Kip1}$ concentrations. There was no significant difference in the levels of cyclin D1 expression in high versus low expressors of cyclin E. As cyclin E expression reached high levels ($> 40\%$), its association with the proliferation index changed from positive to negative, and this was accompanied by an increase in $p27^{Kip1}$ expression, with no significant change in cyclin D1. Taken together, these findings suggest that the inverse relation between cyclin E and the proliferation index in high expressors of cyclin E might be explained, at least in part, by a coordinate increase in $p27^{Kip1}$ mediated inhibition of cyclin E activity.

Our study supports the experimental data of Sgambato *et al*.[43] They demonstrated that ectopic overexpression of cyclin E in the HC11 mouse mammary epithelial cell line resulted in a prolonged G1 phase and a protracted G0–S transition, leading to growth inhibition. This was accompanied by increased $p27^{Kip1}$ and decreased cyclin E associated kinase activity. They suggested a putative positive feedback loop between cyclin E and $p27^{Kip1}$.

Del Pizzo and colleagues[22] reported that in primary bladder TCC, $\geq 30\%$ expression of cyclin E was associated with prolonged survival, whereas $< 30\%$ expression was associated with reduced survival. Our data suggest that the reported prognostic significance of cyclin E may be the result of its functional inactivation by the increasing amounts of $p27^{Kip1}$ seen in high expressors of cyclin E. In contrast, cyclin

Take home messages

- Our findings suggest that cyclins D1 and E and p27^{Kip1} play a role in the proliferation, differentiation, and progression of bladder transitional cell carcinomas (TCCs)
- Cyclins D1 and E may cooperate in driving proliferation in the early stages of tumour progression
- Cyclin E may be important in bladder cancer progression
- These findings have implications for the development of novel therapeutic strategies targeting G1 phase cell cycle proteins in TCC

E is probably functionally active in low expressors, as demonstrated by a positive correlation between its level of expression and the proliferation index.

The relation between p27^{Kip1} expression and the proliferation index has not previously been studied in TCC. In normal cells, there is an inverse relation between p27^{Kip1} expression and the proliferation index.[44 45] However, in tumour cells this association is not always present. In breast[46] and colorectal cancers,[47] p27^{Kip1} expression does not correlate with the proliferation index, whereas in some other tumours, an inverse association has been reported[45 48 49] We found a weak but significant inverse relation between p27^{Kip1} expression and proliferation index in primary bladder cancer. Moreover, similar to previous reports in some cancers,[41 44 47] we identified a subset of TCCs with high proliferation indices despite high level expression of p27^{Kip1}. High concentrations of p27^{Kip1} are normally characteristic of quiescent cells, so it is not clear how these rapidly proliferating tumours tolerate this.

Concurrent expression of cyclins D1 and E and the CDKI, p27^{Kip1}, has not previously been reported in TCC. We have elucidated important interrelations between the expression of these proteins and the proliferation index that support a role for them in tumour proliferation, differentiation, and progression. Our results (1) indicate that cyclins D1 and E may cooperate in driving proliferation in the early stages of tumour progression; (2) suggest that cyclin E may be important in bladder cancer progression, despite the lack of a significant correlation between its level of expression and tumour grade or pathological stage; and (3) underscore the importance of studying cell cycle proteins concurrently rather than independently. These findings have implications for the development of novel therapeutic strategies targeting G1 phase cell cycle proteins in TCC.

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ECHO

DNA protein crosslinks and p53 expression are important in defining the danger of exposure to formaldehyde

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DNA protein crosslinks (DPC) and mutation in the p53 tumour suppressor gene may be causally related and represent steps in the carcinogenicity of formaldehyde (FA). This has important implications for workers exposed to the latter.

Formaldehyde resins are used in producing adhesives for wood, plastics, textile, and leather while FA itself is a bactericide and tissue preservative. It is known to be a carcinogen; in vitro and in mammalian studies its primary genotoxic effect is the formation of DPC in target tissues but no studies on the latter had been conducted previously on humans.

The investigators examined DPC in peripheral blood mononuclear cells and p53 in serum from 186 workers in hospital laboratories ('exposed') and 213 hospital administrators ('controls').

DPC levels were significantly higher in the exposed group and the mean amount increased with increasing levels of exposure to FA, as determined by ambient air analysis. Additionally FA exposure increased the risk of having pantropic p53>150 pg/ml and mutant p53.

As FA undergoes exceptionally rapid biotransformation once absorbed there is no reliable direct biomarker. Thus, measuring DPC can be regarded as a surrogate for the dose of FA at critical target sites. The implications of this study are to provide a biologically plausible explanation for the epidemiological evidence of cancer risk from FA; and it may provide a method for screening exposed workers to identify those at higher risk.

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