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 p27Kip1 allow uncontrolled tumour cell proliferation. This study investigated the relationship between these three cell cycle proteins and tumour proliferation in bladder cancer.

Method: Nuclear expression of cyclin D1, cyclin E, and p27Kip1 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 p27Kip1 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 (> 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 p27Kip1 expression. Overall, there was an inverse association between p27Kip1 expression and proliferation. However, a subset of tumours displayed high proliferation indices despite high p27Kip1 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 p27Kip1 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. The activity of the cyclin–CDK complexes is negatively regulated by the CDK inhibitors. 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. Not surprisingly, regulatory defects involving the G1–S checkpoint have been observed in many human cancers.

"The cyclin dependent kinase (CDK) inhibitor, p27Kip1 blocks the activity of cyclin D1–CDK4/6 and cyclin E–CDK2, and is an important negative regulator of the G1–S transition"
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 H2O2 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 p27kip1 and Ki-67 expression in lymphocytes were included as positive controls for p27kip1 and Ki-67. In addition, stromal or peritumoural lymphocytes were present in most cases and served as internal controls for p27kip1 and Ki-67. As previously reported by Dutta et al., 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, p27kip1, 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 = 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 p < 0.05. Statistical analyses were performed on Stata 6.0 (Stata Corporation, College Station, Texas, USA).

RESULTS
Expression of cyclin D1, cyclin E, p27Kip1, and Ki-67
Figures 1 and 2 show typical examples of the immunohistochemical expression of cyclin D1, cyclin E, p27Kip1, 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%
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.

p27Kip1 nuclear reactivity ranged from 0% to 67.4% in the tumour cells. Less than 30%, 30–50%, and >50% p27Kip1 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 p27Kip1 (<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 p27Kip1 expression, which was seen in 12 of 50 cases. The combination closest to a normal profile—low cyclin D1 and E expression but high p27Kip1 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 p27Kip1.

**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 (≥pT2) and non-papillary tumours than in their superficial and papillary counterparts (p = 0.002 and 0.03, respectively; table 1). In contrast, p27Kip1 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 p27Kip1. Cyclin D1 was also strongly associated with papillary morphology (p < 0.0001), whereas p27Kip1 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, p27Kip1, 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 (as determined by Ki-67 expression) and cyclin D1 expression were notably more significant for cyclin D1 than for p27Kip1. These correlations were reversed in low expressors of cyclin D1 (<5% nuclei positive, n = 31) versus high 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 = -0.68, p = 0.002; fig 3D). In contrast, this association was reversed in low expressors of cyclin E (r = 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 p27Kip1, a major inhibitor of cyclin E-associated kinase activity. We found that high expressors of cyclin E (≥30%) compared with low expressors had significantly higher levels of p27Kip1 expression (mean, 35.0%; SEM, 4.5%; and pathohistological variables

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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).
displayed a positive correlation between p27Kip1 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 p27Kip1, thereby decreasing the pool of p27Kip1 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 p27Kip1 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 p27Kip1 ($>30$%). 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 p27Kip1 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 $r_s = -0.15$, $p = 0.534$;
DISCUSSION

Overexpression of the G1 cyclins, D1 and E, and/or down-regulation of p27Kip1 are potent mechanisms that allow tumour cells to undergo uncontrolled proliferation.24 In normal tissues, concentrations of p27Kip1 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 p27Kip1, 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 p27Kip1 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 p27Kip1 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 down-regulation 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. 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 31–35 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
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. 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 p27Kip1 expression was positively correlated with the Ki-67 proliferation index in all TCCs, but most strongly in the superficial but not muscle invasive TCCs. Positive correlations were found between high levels of cyclin E expression and the proliferation index in superficial versus muscle invasive TCCs. The ectopic expression of both cyclins D1 and E can effectively sequester p27Kip1 away from cyclin E–CDK2 complexes, so that changes in the level of cyclin D1 can result in altered cyclin E associated kinase activity, without a corresponding change in p27Kip1 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 proliferative index changed from positive to negative, and this was accompanied by an increase in p27Kip1 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 p27Kip1 mediated inhibition of cyclin E activity.

Our study supports the experimental data of Sgambato et al. 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 p27Kip1 and decreased cyclin E associated kinase activity. They suggested a putative positive feedback loop between cyclin E and p27Kip1.

Del Pizzo and colleagues reported that in primary bladder TCC, 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 p27Kip1 seen in high expressors of cyclin E. In contrast, cyclin

**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 p27Kip1 expression (level of p27Kip1 expression subtracted from G1 cyclin index), showing a positive correlation. (D) Superficial tumours only, showing an even stronger positive correlation (r<sub>S</sub>, Spearman’s rank correlation coefficient). Filled and unfilled circles represent superficial and muscle invasive tumours, respectively.
E is functionally active in low expressors, as demonstrated by a positive correlation between its level of expression and the proliferation index.

The relation between p27Kip1 expression and the proliferation index has not previously been studied in TCC. In normal cells, there is an inverse relation between p27Kip1 expression and the proliferation index. However, in tumour cells this association is not always present. In breast and colorectal cancers, p27Kip1 expression does not correlate with the proliferation index, whereas in some other tumours, an inverse association has been reported. We found a weak but significant inverse relation between p27Kip1 expression and proliferation index in primary bladder cancer. Moreover, similar to previous reports in some cancers, we identified a subset of TCCs with high proliferation indices despite high level expression of p27Kip1. High concentrations of p27Kip1 are normally characteristic of quiescent cells, so it is not clear how these rapidly proliferating tumours tolerate this.

Concurrent expression of cyclin D1 and E and the CDK1, p27Kip1, 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.

**Take home messages**

- Our findings suggest that cyclins D1 and E and p27Kip1 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.

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**REFERENCES**

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

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 panoptic 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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