Expression of G1→S Transition Regulatory Molecules in Human Urothelial Cancer

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Growth of cancer cells is characterized by accelerated passage through the cell cycle, which is often caused by deregulation of the G1→S transition. In this study the expression of G1→S transition regulatory molecules was analyzed in 32 transitional cell carcinoma specimens and fifteen normal tissues obtained by cystectomy or nephroureterectomy of mainly locally advanced tumors, as well as six bladder cancer cell lines. Expression of mRNAs for cyclins D1 and D2 and cyclin-dependent kinases (CDK) 2 and 4 was investigated by quantitative reverse transcription-polymerase chain reaction. Overexpression of cyclin D1 compared to normal mucosa was observed in 3 tumors (9.4%), but in neither of the cell lines. All tumors with overexpression were moderately differentiated (G2) pT1 or pT2 tumors, and thus among the less advanced specimens. Cyclin D2 was not expressed in normal bladder mucosa or in tumors. The expression of CDK4 mRNA varied within the same range in mucosa, tumors, and cell lines. CDK2 mRNA expression varied more strongly and was diminished in individual tumors and in four cell lines. It is concluded that cyclin D1 overexpression can play an important role in the early stage of urothelial tumorigenesis, driving cell proliferation. Ectopic expression of cyclin D2 or amplification of CDK4 does not occur at a significant frequency in urothelial carcinomas. Different expression patterns of cyclin D1 and CDK2 indicate heterogeneity in the mechanisms of G1→S transition deregulation in individual bladder tumors which may elicit differences in their biological and clinical behavior.

Key words: Cyclin D — CDK4 — CDK2 — RT-PCR — Rb

At the G1→S transition of the cell cycle, it is decided whether cells continue to progress through the cell cycle or become quiescent. This decision is controlled in part by extracellular factors stimulating or inhibiting growth through regulation of cyclins and cyclin-dependent kinases (CDK). The expression of D cyclins is dependent on growth factors.10 The D cyclins associate with and activate the CDK4 and CDK6.2, 3 These kinases phosphorylate the retinoblastoma protein Rb. A second phosphorylation by the cyclin E/CDK2 complex then inactivates Rb, thereby releasing transcription factors such as E2F-1, which initiate transcription of genes required for DNA replication.4 Deranged expression of G1/S transition regulatory proteins can therefore underlie altered proliferation of cancer cells.

The human gene encoding cyclin D1, located on chromosome 11q13, was first isolated from human parathyroid adenomas as the PRAD1 gene translocated to the parathyroid hormone (PTH) locus at 11p15.5, 6 It is identical to the BCL-1 proto-oncogene which is translocated and activated in a subset of B-cell lymphomas.7, 8 Chromosome 11q13 is frequently amplified in a subset of human solid tumors.10–12 Amplification or overexpression of cyclin D1 has been demonstrated in a variety of solid cancers, including esophageal,13 breast,14 colorectal15 and hepatocellular16 cancers. Transfection experiments have shown overexpression of cyclin D1 cDNA to elicit or facilitate cell transformation.17, 18 These findings suggest that cyclin D1 may act as an oncogene. Gene amplification in 11q13 has been reported in 5–20% of urothelial cancers.19, 20 Lee et al. observed cyclin D1 overexpression only in papillary and in low grade tumors (pTa or pT1).21 Bringuier et al. reported fair to high expression of cyclin D1 protein even in bladder tumors lacking amplification of the gene.22 Thus, it is not clear how amplification relates to the expression of cyclin D1. Since amplification translates into increased protein levels via an increase in mRNA, an evaluation of cyclin D1 mRNA levels appeared useful to clarify this question.

A closely related G1 cyclin, cyclin D2, is overexpressed in murine leukemia virus-induced T-cell leukemia due to a proviral insertion.23 In humans, cyclin D2 is specifically expressed in testicular tissues as well as in male germ cell tumors.24 Moreover, ectopic expression was observed in various human cancer cell lines.25 Thus, ectopic expression of cyclin D2 may contribute to tumorigenesis in different human solid tumors.

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Overexpression of cyclins increases the concentration of active CDK/cyclin complexes in the cell, thereby stimulating the G1→S transition of the cell cycle. Therefore, overexpression of CDKs in gliomas and sarcomas could constitute an alternative mechanism promoting inappropriate cell proliferation. Expression of CDK mRNAs has not yet been investigated in bladder cancer. To obtain a more comprehensive picture of the expression of these molecules we have investigated the mRNA levels of the G1→S transition regulators cyclin D1 and D2 and CDK2 and 4 in a series of 32 urothelial cancers and in six transitional cell carcinoma (TCC) cell lines by quantitative reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Specimens. The tissue specimens were obtained from 31 patients undergoing cystectomy and one patient (#13) undergoing nephroureterectomy for TCC. Tumor regions and normal adjacent mucosal tissues were identified by a pathologist immediately after surgery, frozen in liquid nitrogen and stored at −80°C. Patient data and tumor characteristics are listed in Table I. The mean age of the patients was 66.1 ± 9.3 (mean±SD); 24 patients were male and 7 were female. Grading and the staging were performed according to the TNM classification (UICC). Two tumors were staged as pTa, one as pT1, five as pT2, four as pT3a, sixteen as pT3b, and four as pT4. Tumor grading was G2 in 10 and G3 in 22 cases.

Cell lines. Six human bladder cancer cell lines, J82, VMClbIII, T24, 647V, 5637, HT1376 were cultured as described previously. Relevant properties are summarized in Table II. The Rb status as determined by western blotting and DNA analysis was previously reported.

RNA extraction. Total RNA was prepared from preconfluent cell monolayers or frozen tissue by guanidinium/acid phenol/chloroform extraction (TRIZolReagent) as recommended by the supplier (Life Technologies, Berlin, Germany). Following re-extraction with chloroform and precipitation with isopropanol, RNA was redissolved in diethyl pyrocarbonate-treated (DEPC) water and quantitated by spectrophotometry.

Reverse transcription and PCR. Analysis of mRNA levels was performed essentially as described. Two micrograms of RNA from each sample was reverse-transcribed using oligo (dT) primer (Sigma, München, Germany) and avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI). PCR amplification was carried out using PrimeZyme II DNA polymerase (Biometra, Göttingen, Germany) in the presence of 2.5 µM digoxigenin-labelled dUTP (Boehringer-Mannheim, Mannheim, Germany) under standard conditions with 20 pmol each of sense and antisense primers. The number of PCR cycles was kept within the linear range of amplification, which was separately determined for each cDNA. Primer sequences and amplification conditions are given in Table III. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers have been provided.

Table I. Summary of Tumor Characteristics

| Patient | Age | Gender | Stage | Lymphnode | Grade |
|---------|-----|--------|-------|-----------|-------|
| 1       | 78  | f      | pT3b  | pN0       | 3     |
| 2       | 74  | m      | pT4   | pN1       | 3     |
| 3       | 60  | m      | pT3b  | pN0       | 3     |
| 4       | 61  | m      | pT3b  | pN0       | 3     |
| 5       | 57  | m      | pT2   | pN0       | 2     |
| 6       | 62  | m      | pT3a  | pN0       | 2     |
| 7       | 61  | m      | pT3b  | pN0       | 3     |
| 8       | 84  | f      | pT2   | pN0       | 3     |
| 9       | 67  | m      | pT3b  | pN2       | 2     |
| 10      | 60  | f      | pT3b  | pN2       | 3     |
| 11      | 73  | m      | pT3a  | pN0       | 3     |
| 12      | 85  | m      | pT2   | pN0       | 2     |
| 13      | 70  | m      | pT3b  | pN0       | 2     |
| 14      | 60  | m      | pT1   | pN0       | 2     |
| 15      | 74  | m      | pT3b  | pN0       | 2     |
| 16      | 76  | f      | pT3b  | pN1       | 3     |
| 17      | 73  | m      | pT3b  | pN0       | 3     |
| 18      | 73  | m      | pT3b  | pN0       | 2     |
| 19      | 62  | m      | pT2   | pN0       | 3     |
| 20      | 68  | m      | pT3b  | pN2       | 3     |
| 21      | 65  | m      | pT3b  | pN2       | 3     |
| 22      | 68  | m      | pT3b  | pN0       | 3     |
| 23      | 46  | f      | pT3a  | pN0       | 2     |
| 24      | 66  | m      | pT3b  | pN2       | 3     |
| 25      | 65  | m      | pT3a  | pN0       | 3     |
| 26      | 76  | m      | pT4   | pN1       | 3     |
| 27      | 45  | m      | pT4   | pN0       | 3     |
| 28      | 68  | m      | pT3a  | pN1       | 3     |
| 29      | 59  | m      | pT4   | pN0       | 3     |
| 30      | 64  | f      | pT3a  | pN0       | 3     |
| 31      | 55  | m      | pT2   | pN1       | 2     |
| 32      | 60  | m      | pT3b  | pN0       | 3     |

Table II. Properties of Bladder Cancer Cell Lines

| Cell line | Rb protein | Cyclin D1 | CDK4 | CDK2 |
|-----------|------------|-----------|------|------|
| J82       | truncated  | 40        | 49   | 25   |
| VMClbIII  | intact     | 177       | 190  | 25   |
| T24       | intact     | 125       | 101  | 6    |
| 647V      | deleted    | 138       | 278  | 380  |
| 5637      | deleted    | 179       | 163  | 95   |
| HT1376    | deleted    | 64        | 185  | 550  |

a) Relative levels of mRNA compared to the mean value of normal mucosa set as 100%.
Each reaction was begun with an initial cycle of 5 min denaturation at 96°C, 5 min at the specific annealing temperature, and 90 s extension at 72°C, followed by definite cycles of 96°C for 30 s, the specific annealing temperature for 45 s, and 72°C for 90 s. The final extension was performed at 72°C for 10 min. PCR products were separated on a 2% agarose gel before overnight transfer to a nylon membrane (Hybond-N+, Amersham, Buckinghamshire, UK). Following detection with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim), luminogram signals were quantitated from appropriately exposed films by video densitometry (ONE-D-SCAN 1.0, Scanalytics, Billerica, MA). Values were related to GAPDH and are expressed as relative expression units (rU) calculated as G1→S transition regulator signal/GAPDH signal.

### RESULTS

**Cyclin D1 and D2 mRNA expression in urothelial tumors** Cyclin D1 mRNA expression was determined in 32 transitional cell carcinomas and 15 samples of normal urothelial mucosa (Fig. 1A). Although the mean value of cyclin D1 expression was higher in tumors (1.00 ± 0.99 rU) than in normal tissue (0.61 ± 0.47 rU), the difference was statistically not significant. However, in three patients (8, 12 and 14) cyclin D1 mRNA expression in the tumor samples dramatically exceeded that in normal mucosa (Fig. 1A). All three were moderately differentiated (G2) pT1 or pT2 tumors (Table I).

No cyclin D2 expression was detected by RT-PCR in two samples of normal mucosa and eight tumor specimens selected at random. Expression of this cyclin was easily detected in normal and malignant testicular tissues used as positive controls. A conservative estimate of the detection limit of the RT-PCR for cyclin D2 is 5% of the expression level in normal testes. Therefore, the expression of cyclin D2 in TCC specimens did not exceed this value.

**Expression of CDKs in urothelial tumors** Expression of CDK4 mRNA was determined in 29 tumors and 10 samples of normal mucosa (Fig. 1B). Almost identical expression levels were found in tumor tissue and in normal tissue (0.76 ± 0.35 rU in tumors vs. 0.82 ± 0.29 rU in normal tissue). None of the tumors showed a significant increase in CDK4 expression. A few tumors displayed decreased expression, notably #18, a pT3b G2 specimen.

Expression of CDK2 mRNA determined in the same 29 tumors and 10 samples of normal mucosa was more variable than that of CDK4 (Fig. 1C), but on average, expression of CDK2 was very similar in cancer and normal tissues (0.19 ± 0.14 rU in tumors vs. 0.20 ± 0.11 rU in normal tissues). Several advanced-stage carcinomas exhibited decreased expression of CDK2, notably #8, 18, 25, 29.

**Expression of G1→S transition molecules in bladder cancer cell lines** Since expression of cyclin D1 mRNA has been reported to depend on the RB protein status, it was compared in cell lines with intact or mutated RB protein (Table II). In two RB-defective cell lines, 5637 and 647V, cyclin D1 mRNA expression in the tumors dramatically exceeded that in normal mucosa (Fig. 1A). All three were moderately differentiated (G2) pT1 or pT2 tumors (Table I).

No cyclin D2 expression was detected by RT-PCR in two samples of normal mucosa and eight tumor specimens selected at random. Expression of this cyclin was easily detected in normal and malignant testicular tissues used as positive controls. A conservative estimate of the detection limit of the RT-PCR for cyclin D2 is 5% of the expression level in normal testes. Therefore, the expression of cyclin D2 in TCC specimens did not exceed this value.

### Table III. Primer Sets and Conditions for PCR Analyses

| Gene     | Sequence of primers (sense/antisense) | Annealing temperature (°C) | Cycles | Amplicon size (base pairs) |
|----------|--------------------------------------|-----------------------------|--------|---------------------------|
| Cyclin D1| 5′-GAGACCATCCCCCTGACGGC-3′<br>5′-TCTTCCTCTCCTCGGCGC-3′ | 65                            | 22     | 485                       |
| Cyclin D2| 5′-TGCATGTTCCTCGGCCCTCC-3′<br>5′-TTAAAGTCCCGTGACACA-3′ | 65                            | 17     | 247                       |
| CDK2     | 5′-GCTTTTCTGCCATTCTCATCG-3′<br>5′-GTCCCCAAGCTCCGAAAGAT-3′ | 57                            | 22     | 317                       |
| CDK4     | 5′-TGTTGTGCCGTCTGATTTGGA-3′<br>5′-ACCGGGTGAAGTCGCCATCTG-3′ | 59                            | 20     | 486                       |
| GAPDH    | 5′-TCCCATCACCACATCTCCCA-3′<br>5′-CATCAGCCACAGTTTCC-3′ | 61                            | 17     | 380                       |

G1→S Transition Molecules in TCC
Fig. 1. Expression of G1/S transition regulatory molecules in TCC. Expression levels of mRNAs for cyclin D1 (A), CDK4 (B), and CDK2 (C) relative to GAPDH mRNA are shown. The solid lines indicate the mean expression levels and the broken lines the standard deviations, respectively, in normal mucosa. Note that tumors are ordered from left to right by increasing stage and grade.
cell lines (647V, HT1376) showed increased expression (Fig. 3). This difference was not related to the Rb status.

DISCUSSION

Aberrant expression of cyclin D1 has been shown to contribute to tumorigenesis in several human tumors. In TCC, amplification of the chromosomal region 11q13 \textsuperscript{19,20} as well as overexpression of cyclin D1 protein \textsuperscript{22} have been reported. However, the relationship between cyclin D1 expression and tumor progression is complex. Using immunohistochemistry, Lee \textit{et al.} \textsuperscript{21} observed cyclin D1 overexpression to be confined to pTa and pT1 tumors. In accord with these data, Bringuier \textit{et al.} detected low expression of cyclin D1 protein in a more aggressive subset of bladder tumors. \textsuperscript{23} In our series, cyclin D1 mRNA was overexpressed in three of 32 (9.4%) carcinomas. Notably, all overexpressing tumors were G2 carcinomas staged pT1 or pT2, thus belonging to the few early-stage specimens in our series (Fig. 1A). It therefore appears that cyclin D1 overexpression is inversely correlated with tumor aggressiveness, suggesting that cyclin D1 overexpression may play an important role in the early stage of bladder tumorigenesis, but is usually lost during tumor progression and dissemination. Since cyclin D1 expression in normal proliferating cells is dependent on the presence of growth factors, \textsuperscript{1,35,36} it is conceivable that the overexpression of the gene in early-stage tumors reflects residual growth regulation, as recently suggested for p21\textsuperscript{WAF1} mRNA, which similarly is increased in early-stage tumors and lost in most advanced specimens. \textsuperscript{37} Overexpression of cyclin D1 may persist in advanced tumors only in the case of gene amplification. \textsuperscript{20} Cyclin D1 overexpression has been shown to enhance cell proliferation by accelerating Rb phosphorylation, but does not obliterate the G1→S restriction point entirely, as does
expression or deletion of the Rb gene in advanced TCC. Furthermore, from a detailed investigation of chemically induced rat bladder tumors, there is evidence that cyclin D1 expression in urothelial tissue may support not only cell proliferation, but also differentiation.

Ectopic expression of cyclin D2, found in several human solid tumors, represents an alternative to overexpression of cyclin D1 for deregulation of the G1→S transition. However, cyclin D2 was not detected even by RT-PCR in TCC samples in this study and therefore apparently does not contribute to urothelial carcinogenesis.

CDK4 expression was found to be unaltered in almost all tumor tissues, as well as in cell lines. In particular, no instance of overexpression was observed. This suggests that deregulation of CDK4 expression by amplification of the 12q amplicon containing the CDK4 gene is rare in urothelial cancer. Rather, the relatively narrow range of expression found in both normal tissue and tumor samples suggests that CDK4 expression in urothelium is constitutive.

CDK2 expression varied more strongly between tumor samples and urothelial carcinoma cell lines. Since CDK2 mRNA expression is significantly decreased in human germ cell tumors [Schmidt et al., submitted for publication], it is possible that CDK2 gene expression may not be entirely constitutive. Since many human germ cell tumors lack Rb protein, the correlation between Rb status and CDK2 mRNA expression was investigated in bladder carcinoma cell lines whose Rb status is known. Decreased expression of CDK2 did not correlate with the presence of intact Rb protein. The observed decreases in CDK2 or CDK4 expression in individual tumors could conceivably result from loss of chromosome 12p or 12q, respectively, where these genes are located. Loss of 12p in particular has been reported in advanced urothelial cancers.

Recently, the Rb protein has been reported to activate the cyclin D1 promoter. Therefore, loss of Rb ought to result in down-regulation of cyclin D1 transcription. Nevertheless, the four bladder cancer cell lines with defective Rb showed similar expression of cyclin D1 to the two other cell lines with intact Rb. This is in contrast to a report by Müller et al. showing a significant decrease of cyclin D1 expression in cell lines originating from various tissues, but not bladder, with defective Rb.

Summarizing the findings of this study, it appears that in TCC cyclin D1 overexpression is frequent in low-stage and low-grade tumors, but is lost during progression. Decreased expression of CDK2 occurs in individual advanced tumors. Overexpression of CDK4 and ectopic expression of cyclin D2 do not play a major role in TCC. The different expression patterns of G1/S transition regulatory molecules in individual urothelial tumors may reflect differences in the mechanisms of tumorigenesis and elicit differences in their biological and clinical behavior.

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REFERENCES

1) Matsushime, H., Roussel, M. F., Ashmun, R. A. and Sherr, C. J. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell, 65, 701–703 (1991).
2) Bates, S., Bonetta, L., MacAllan, D., Parry, D., Holder, A., Dickson, C. and Peters, G. Cdk6 (PLSTIRE) and Cdk4 (PSK-13) are distinct subsets of the cyclin-dependent kinases that associate with cyclin D1. Oncogene, 9, 71–79 (1994).
3) Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E. and Sherr, C. J. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev., 7, 331–342 (1993).
4) Sala, A., Nicolaides, N. C., Engelhard, A., Bellon, T., Lawo, D. C., Arnold, A., Gnara, X., Giordano, A. and Calabretta, B. Correlation between E2F-1 requirement in the S phase and E2F-1 transactivation of cell cycle-related genes in human cells. Cancer Res., 54, 1402–1406 (1994).
5) Rosenberg, C. L., Kim, H. G., Shows, T. B., Kronenberg, H. M. and Arnold, A. Rearrangement and overexpression of D11S287E, a candidate oncogene on chromosome 11q13 in benign parathyroid tumors. Oncogene, 6, 449–454 (1991).
6) Motokura, T., Bloom, T., Kim, H. G., Jüppner, H., Ruderman, J. V., Kronenberg, H. M. and Arnold, A. A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature, 350, 512–515 (1991).
7) Rosenberg, C. L., Wong, E., Petty, E. M., Bale, A. E., Tsujimoto, Y., Harris, N. L. and Arnold, A. PRAD1, a candidate BCL1 oncogene: mapping and expression in centrocytic lymphoma. Proc. Natl. Acad. Sci. USA, 88, 9638–9642 (1991).
8) Withers, D. A., Harvey, R. C., Faust, J. B., Melnyk, O., Carey, K. and Meeker, T. C. Characterization of a candidate bcl-1 gene. Mol. Cell. Biol., 11, 4846–4853 (1991).
9) de Boer, C. J., Loysen, S., Kluij, P. M., Kluij-Nellemans, H. C., Schuurung, E. and van Krieken, J. H. Multiple breakpoints within the BCL-1 locus in B-cell lymphoma: rearrangements of the cyclin D1 gene. *Cancer Res.*, **53**, 4148–4152 (1993).

10) Lammie, G., Fantl, V., Smith, R., Schuurung, E., Brookes, S., Michalides, R., Dickson, C., Arnold, A. and Peters, G. D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. *Oncogene*, **6**, 439–444 (1991).

11) Schuurung, E., Verhoeven, E., Mooi, W. J. and Michalides, L. A. M. Identification and cloning of two overexpressed genes, U21B31/PRAD1 and EMS1, within the amplified chromosome 11q13 region in human carcinomas. *Oncogene*, **7**, 355–361 (1992).

12) Theilllet, C., Adnane, J., Zepetowski, P., Simon, M. P., Jeanteur, P., Birnbaum, D. and Gaudray, P. BCL-1 participates in the 11q13 amplification found in breast cancer. *Oncogene*, **5**, 147–149 (1990).

13) Jiang, W., Zhang, Y.-J., Kahn, S., M. Hollstein, M. C., Santella, R. M., Lu, S.-H., Harris, C. C., Montesano, R. and Weinstein, I. B. Altered expression of the cyclin D1 and retinoblastoma genes in human esophageal cancer. *Proc. Natl. Acad. Sci. USA*, **90**, 9026–9030 (1993).

14) Buckley, M. F., Sweeney, K. J. E., Hamilton, J. A., Sini, R. L., Manning, D. L., Nicholson, R. I., deFazio, A., Watts, C. K. W., Musgrove, E. A. and Sutherland, R. L. Expression and amplification of cyclin genes in human breast cancer. *Oncogene*, **8**, 2127–2133 (1993).

15) Leach, F. S., Elledge, S. J., Sherr, C. J., Wilson, J. K. V., Markowitz, S., Kinzler, K. W. and Vogelstein, B. Amplification of cyclin genes in colorectal carcinomas. *Cancer Res.*, **53**, 1986–1989 (1993).

16) Zhang, Y.-J., Jiang, W., Chen, C. J., Lee, C. S., Kahn, S. M., Santella, R. M. and Weinstein, I. B. Amplification and overexpression of cyclin D1 in human hepatocellular carcinoma. *Biochem. Biophys. Res. Commun.*, **196**, 1010–1016 (1993).

17) Lovec, H., Sewing A., Lucibello, F. C., Muller, R. and Moroy, T. Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras: link between cell cycle control and malignant transformation. *Oncogene*, **9**, 323–326 (1994).

18) Hinds, P. W., Dowdy, S. F., Eaton, E. N., Arnold, A. and Weinberg, R. A. Function of a human cyclin gene as an oncogene. *Proc. Natl. Acad. Sci. USA*, **91**, 709–713 (1994).

19) Tsutsumi, M., Sakamoto, H., Yoshida, T., Kakizoe, T., Koiso, K., Sugimura, T. and Terada, M. Coamplification of the hst-1 and int-2 genes in human cancers. *Jpn. J. Cancer Res.*, **79**, 428–432 (1988).

20) Proctor, A. J., Coombs, J. M., Cairns, J. P. and Knowles, M. A. Amplification at chromosome 11q13 in transitional cell tumors of the bladder. *Oncogene*, **6**, 789–795 (1991).

21) Lee, C. C. R., Yamamoto, S., Morimura, K., Waniubachi, H., Nishisaka, N., Ikemoto, S., Nakatani, T., Wada, S., Kishimoto, T. and Fukushima, S. Significance of cyclin D1 overexpression in transitional cell carcinomas of the urinary bladder and its correlation with histopathologic features. *Cancer*, **79**, 780–789 (1997).

22) Bringuiar, P. P., Tamimi, Y., Schuurung, E. and Schalken, J. Expression of cyclin D1 and EMS1 in bladder tumors; relationship with chromosome 11q13 amplification. *Oncogene*, **12**, 1747–1753 (1996).

23) Hanna, Z., Jankowski, M., Tremblay, P., Jiang, X., Milotovich, A., Franke, U. and Jolicour, P. The Vin-1 gene, identified by provirus insertional mutagenesis, is the cyclin D2. *Oncogene*, **8**, 1661–1666 (1993).

24) Houldsworth, J., Reuter, V., Bosl, G. J. and Chaganti, R. S. Aberrant expression of cyclin D2 is an early event in human male germ cell tumorigenesis. *Cell Growth Differ.*, **8**, 293–299 (1997).

25) Lukas, J., Bartkova, J., Welcker, M., Peterson, O. W., Peters, G., Strauss, M. and Bartek, J. Cyclin D2 is a moderately oscillating nucleoprotein required for G1 phase progression in specific cell types. *Oncogene*, **10**, 2125–2134 (1995).

26) Khatib, Z. A., Matsushima, H., Valentine, M., Shapiro, D. N., Sherr, C. J. and Look, T. Coamplification of the CDK4 gene with MDM2 and GLI1 in human sarcoma. *Cancer Res.*, **53**, 5535–5541 (1993).

27) Reifenberger, G., Reifenberger, J., Ichimura, K., Meltzer, P. S. and Collins, V. P. Amplification of multiple genes from chromosomal region 12q13-14 in human malignant gliomas: preliminary mapping of the amphiion shows preferential involvement of CDK4, SAS, and MDM2. *Cancer Res.*, **54**, 4299–4303 (1994).

28) UICC. “TNM Classification of International Union against Cancer” (1992) Springer-Verlag.

29) Grimm, M.-O., Jurgenus, B., Schulz, W. A., Decken, K., Makri, D. and Schmitz-Dräger, B. J. Inactivation of tumor suppressor genes and deregulation of the c-myc gene in urothelial cancer cell lines. *Urol. Res.*, **23**, 293–300 (1995).

30) Horowitz, J. M., Yandel, D. W., Park, S.-H., Canning, S., Whyte, P., Buchkovich, K., Harlow, E., Weinberg, R. A. and Dryja, T. P. Point mutational inactivation of the retinoblastoma anti-oncogene. *Science*, **243**, 937–940 (1989).

31) Horowitz, J. M., Park, S.-H., Bogenmann, E., Cheng, J.-C., Yandel, D. W., Kaye, F. J., Minna, J. D., Dryja, T. P. and Weinberg, R. A. Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. *Proc. Natl. Acad. Sci. USA*, **87**, 2775–2779 (1990).

32) Schmidt, B., Ackermann, R., Hartmann, M. and Strohmeyer, T. Alterations of the metastasis suppressor gene nm23 and the proto-oncogene c-myc in human testicular germ cell tumors. *J. Urol.*, **158**, 2000–2005 (1997).

33) Schoenfeld, A., Luqmani, Y., Smith, D., O’Reilly, S., Shousha, S., Sinnett, H. D. and Coombes, R. C. Detection of breast cancer micrometastases in axillary lymph nodes.
by using polymerase chain reaction. Cancer Res., 54, 2986–2990 (1994).
34) Müller, H., Lukas, J., Schneider, A., Warthoe, P., Bartek, J., Eilers, M. and Strauss, M. Cyclin D1 expression is regulated by the retinoblastoma protein. Proc. Natl. Acad. Sci. USA, 91, 2945–2949 (1994).
35) Won, K. A., Xiong, Y., Beach, D. and Gilman, M. Z. Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. Proc. Natl. Acad. Sci. USA, 89, 9910–9914 (1992).
36) Winston, J. T. and Pledger, W. J. Growth factor regulation of cyclin D1 mRNA expression through protein synthesis-dependent and -independent mechanisms. Mol. Biol. Cell, 4, 1133–1144 (1993).
37) Clasen, S., Schulz, W. A., Gerharz, C. D., Grimm, M. O., Christoph, F. and Schmitz-Dräger, B. J. Frequent and heterogeneous expression of cyclin-dependent kinase inhibitor WAF1/p21 protein and mRNA in urothelial carcinoma. Br. J. Cancer, 74, 515–521 (1998).
38) Cairns, P., Proctor, A. J. and Knowles, M. A. Loss of heterozygosity at the RB locus is frequent and correlates with muscle invasion in bladder carcinoma. Oncogene, 6, 2305–2309 (1991).
39) Lee, C. C. R., Yamamoto, S., Wanibuchi, H., Wada, S., Sugimura, K., Kishimoto, T. and Fukushima, S. Cyclin D1 overexpression in rat two-stage bladder carcinogenesis and its relationship with oncogenes, tumor suppressor genes, and cell proliferation. Cancer Res., 57, 4765–4776 (1997).
40) Strohmeyer, T., Reissmann, P., Cordon-Cardo, C., Hartmann, M., Ackermann, R. and Slamon, D. J. Correlation between retinoblastoma gene expression and differentiation in human testicular tumors. Proc. Natl. Acad. Sci. USA, 88, 6662–6666 (1991).
41) Knowles, M. A., Elder, P. A., Williamson, M., Cairns, J. P., Shaw, M. E. and Law, M. G. Allelotype of human bladder cancer. Cancer Res., 54, 531–538 (1994).
42) Marhin, W. W., Hei, Y. J., Chen, S., Jiang, Z., Gallie, B. L., Phillips, R. A. and Penn, L. Z. Loss of Rb and Myc activation cooperate to suppress cyclin D1 and contribute to transformation. Oncogene, 12, 43–52 (1996).