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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.[1)] 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.[7–9)] 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)] colorectal[15)] and hepatocellular[16)] 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[26, 27] 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).[28] 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, VMCubIII, T24, 647V, 5637, HT1376 were cultured as described previously.[29] Relevant properties are summarized in Table II. The Rb status as determined by western blotting and DNA analysis was previously reported.[29–31]

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.[32] 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

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	pTa	pN0	3
12	85	m	pT2	pN0	2
13	70	f	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	pTa	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[a]	CDK4[a]	CDK2[a]
J82	truncated	40	49	25
VmCubIII	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%.

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′ 5′-TCTTCCTCCTCCTCGGCGGC-3′	65	22	485
Cyclin D2	5′-TGCATGTTCCTGGCCTCC-3′ 5′-TTAAAGTCGGTGGCACACA-3′	65	17	247
CDK2	5′-GCTTTCTGCCATTCTCATCG-3′ 5′-GTCCCCAGAGTCCGAAAGAT-3′	57	22	317
CDK4	5′-TGGTGTCGGTGCCTATGGGA-3′ 5′-ACGGGTGTAAGTGCCATCTG-3′	59	20	486
GAPDH	5′-TCCCATCACCATCTTCCA-3′ 5′-CATCACGCCACAGTTTCC-3′	61	17	380

described.[33] 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 (# 5, 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 mucosa (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 tissue (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,[34] it was compared in cell lines with intact or mutated Rb protein (Table II). In two Rb-defective cell lines, 5637 and 647V and in two cell lines with intact Rb (T24 and VmCubIII), cyclin D1 expression was in the range of normal bladder mucosa samples whereas the other Rb-defective cell lines, J82 and HT1376, showed slightly diminished expression (Fig. 2). CDK4 mRNA expression was almost identical in all six cell lines, whereas CDK2 was differentially expressed. Three of the cell lines (J82, VM CubIII, T24) showed substantially diminished expression of CDK2 compared to normal mucosa and two other

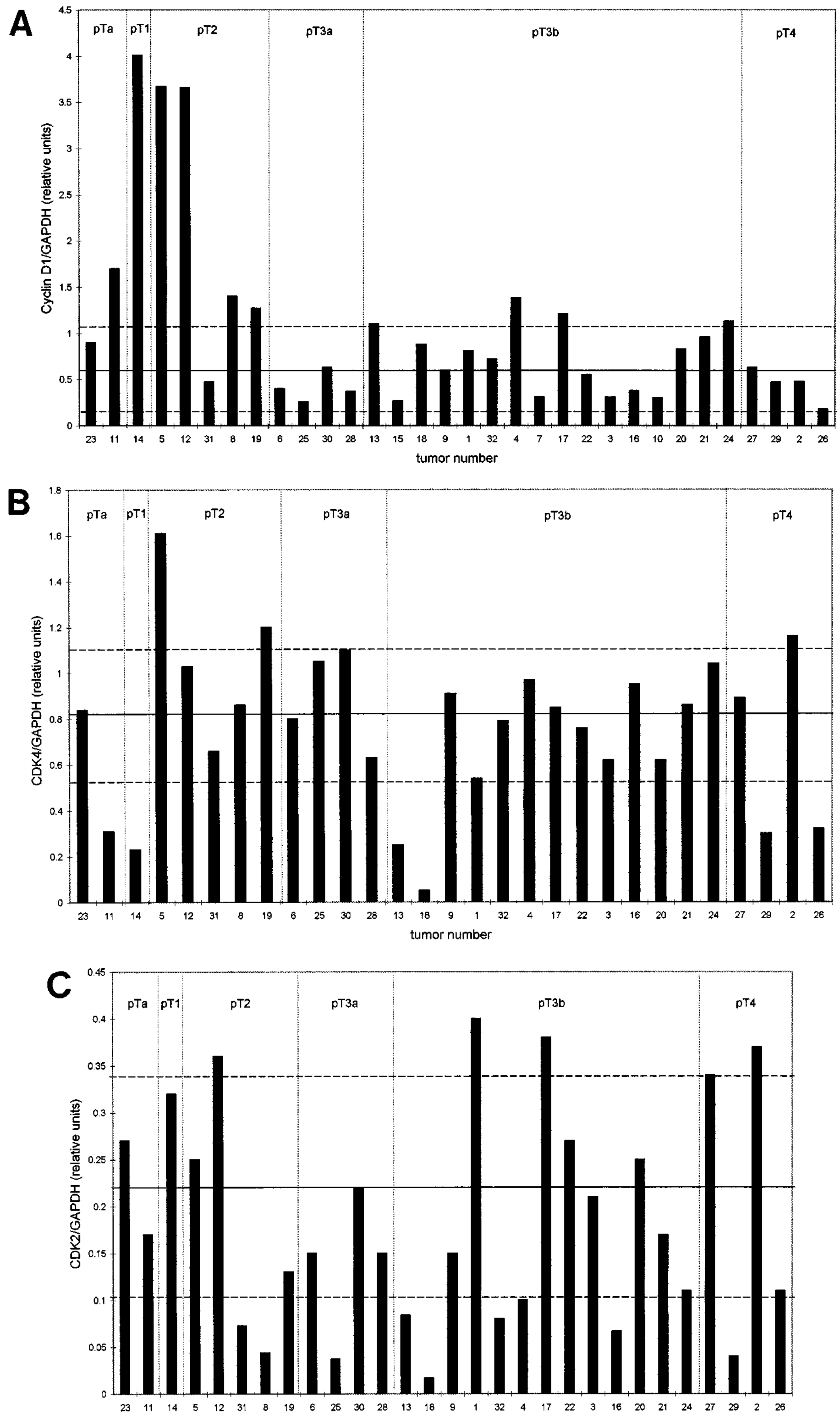

Fig. 1. Expression of G1/S transition regulatory molecules in TCC. Expression levels of mRNAs for cyclin D1 (A), CDK4 (B), and CDK2 (C) relative to GAPDH mRNA are shown. The solid lines indicate the mean expression levels and the broken lines the standard deviations, respectively, in normal mucosa. Note that tumors are ordered from left to right by increasing stage and grade.

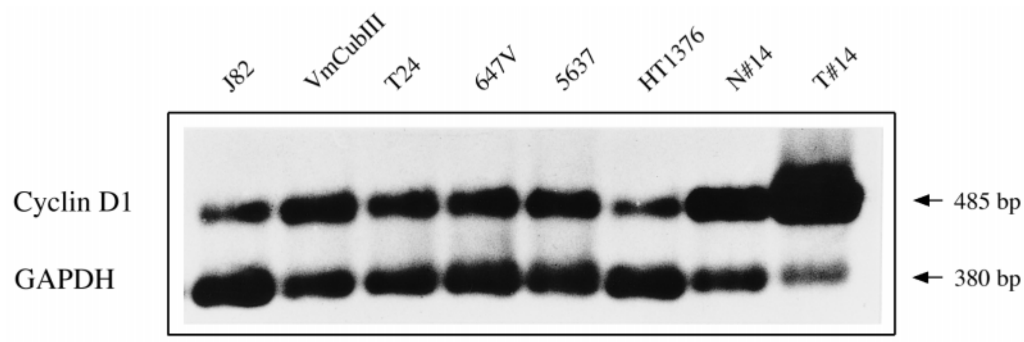

Fig. 2. Expression of cyclin D1 mRNA in bladder carcinoma cell lines. A luminogram from a quantitative RT-PCR experiment using RNA from six cell lines, a normal mucosa (N#14) and the corresponding tumor sample (T#14), which overexpressed cyclin D1, is shown. The sizes of the PCR products are indicated on the right. GAPDH mRNA was used as a control.

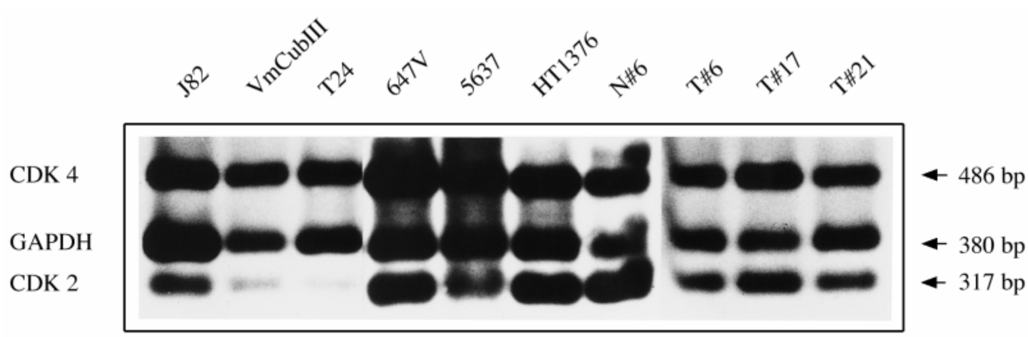

Fig. 3. Expression of CDK2 and CDK4 in bladder carcinoma cell lines. A luminogram from a quantitative RT-PCR experiment using RNA from six cell lines, one normal mucosa (N#6) and three tumor samples (T#14, T#17, T#21) is shown. The sizes of the PCR products are indicated on the right. GAPDH mRNA was used as a control. J82, VmCubIII, T24, 5637, T#6 and T#21 displayed decreased expression of CDK2 compared to normal mucosa (N#6).

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[19, 20] as well as overexpression of cyclin D1 protein[22] have been reported. However, the relationship between cyclin D1 expression and tumor progression is complex. Using immunohistochemistry, Lee *et al.*[21] observed cyclin D1 overexpression to be confined to pTa and pT1 tumors. In accord with these data, Bringuier *et al*. detected low expression of cyclin D1 protein in a more aggressive subset of bladder tumors.[22] 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,[1, 35, 36] it is conceivable that the overexpression of the gene in early-stage tumors reflects residual growth regulation, as recently suggested for $p21^{WAF1}$ mRNA, which similarly is increased in early-stage tumors and lost in most advanced specimens.[37] Overexpression of cyclin D1 may persist in advanced tumors only in the case of gene amplification.[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 mutation or deletion of the *Rb* gene in advanced TCC[38]. Furthermore, from a detailed investigation of chemically induced rat bladder tumors[39] 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,[25] 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,[40] 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.[41]

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.[42] 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*.[34] 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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