LETTER TO THE EDITOR

FLUORESCENCE *IN SITU* HYBRIDIZATION ANALYSIS OF CCND3 GENE AS MARKER OF PROGRESSION IN BLADDER CARCINOMA

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The aim of this study was to assess patterns of *CCND3* gene amplification in bladder cancer and correlate gene status with recurrence-free and progression-free survival. A sequential cohort series of 102 primary bladder tumor samples in which there was enough tissue material to assess CCND3 gene status by fluorescent in situ hybridization (FISH) was the study group. *CCND3 gene* FISH amplification present in 31.4% of bladder carcinomas, was related to tumor progression (p=0.021) and lower time to progression (mean±SD; 25.75±15.25 months) as compared to 33.29±11.0 months in the CCND3 not amplified group (p=0.05). By immunohistochemistry, Cyclin D3 labeling index was higher in the *CCND3* amplified group (mean±SD, 76.69±27.51) than in not amplified (mean±SD, 21.57±7.02) (p<0.0001). The univariate survival analysis showed *CCND3 gene* amplification to be associated to a shorter progression-free survival (p=0.020) together with WHO histological grade (p=0.001) and pT stage category (p<0.0001). Cox's regression analysis selected *CCND3* amplification as an independent predictor of progression-free survival (p= 0.030, RR3.561, 95% CI 1.128-11.236) together with pT category (p<0.0001, RR5.834, 95% CI 2.364-14.395). Our FISH analysis suggests that *CCND3* gene amplification is a marker of aggressiveness and might be a predictor of tumor progression in bladder urothelial carcinoma.

Nearly 80% of patients who initially present with bladder urothelial carcinoma (BC) have tumors confined to the mucosa or submucosa; so called "non muscle invasive" bladder cancer (NMIBC); the

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rest initially present as invasive disease or "muscle-invasive"(MIBC) bladder carcinoma (1-5). The natural history of treated bladder cancer is difficult to predict due to biologic heterogeneity; features that characterize NMIBC are disease recurrence and progression (1-9).

The risk for both recurrence and tumor progression is related to multiple histopathological factors including tumor grade, depth of invasion, tumor size, multiplicity, tumor growth, vascular invasion, and the presence of CIS (1-9). Although these parameters provide some degree of prognostic information, they fail to clearly evaluate each individual tumor's malignant potential. These shortcomings with traditional clinical and histopathological features lead to significant efforts to better define a tumor's true biological potential on a molecular level.

A number of cell cycle aberrations have been studied in an attempt to identify tumors prone to progress (6-16). Tumor proliferation is considered a powerful prognostic indicator of tumor recurrence, and p53 nuclear accumulation is viewed as a marker of progression in bladder tumors; but some studies failed to demonstrate any independent prognostic significance in these patients (6-18). Data concerning other G1-S modulators such as p21Waf1, p27Kip1, and cyclin D1 or apoptotic related markers are limited but of potential interest (6-9).

Alterations of cyclin D3, another upstream regulator of the cell cycle, have been recently reported in bladder cancer (10, 11, 15). Cyclin D3 plays a pivotal role in controlling physiological progression from the G1 to the S phase of the cell cycle (12, 13). Cyclin D3 may be deregulated in a number of human malignant tumors, including lymphoma, breast carcinoma, and melanoma (12, 13). Recent studies suggest that cyclin D3 status might be a marker of response to targeted therapies in neuroblastoma and lung cancer, thus adding interest to studies on cyclin D3 expression in tumors (13, 14, 18).

Cyclin D3 is deregulated in some bladder urothelial carcinomas with data suggesting that cyclin D3 expression could be a marker of aggressiveness (10, 11, 15). *CCND3* gene status has been recently shown to be amplified in urothelial carcinoma in situ (CIS) by fluorescence *in situ* hybridization (FISH), a finding that awaits confirmation in other bladder urothelial carcinomas (16).

The aim of this study was to assess patterns of *CCND3* gene amplification in a consecutive series of newly diagnosed bladder urothelial carcinoma by FISH analysis, and to correlate *CCND3* status with patient's outcome.

MATERIALS AND METHODS

Patients and clinical follow-up

The present study was approved by the committee on ethical standards of the University of Cordoba, (Cordoba, Spain). A sequential cohort series of 102 patients with newly diagnosed bladder cancer was the study group. The patients underwent complete transurethral resection (TURB) and random bladder biopsies and were seen at the University Hospital of Cordoba between the years 2002-2005. Male patients received additional biopsies from the prostatic urethra following standard protocol. A re-TURB was performed when appropriate. Patients showing high grade NMIBC received BCG therapy (Tice strain, 2-5×10 ^{8}colony forming units weekly for 6 consecutive weeks) followed by maintenance for at least one year. Cystectomy was offered to patients with BCG failure and stage category ≥pT2. Patient's follow-up, calculated as the number of months from the date of the diagnostic procedure to the date of the most recent cystoscopy (or to the date of the last visit or death), was (mean ±SD) 29.43±13.09 (range, 3-41) months. Tumor recurrence was defined as reappearance of tumor after the initial treatment with at least one tumor-free cystoscopy. Tumor stage progression was defined as shift to any higher stage category (T1, T2-T4) in recurrent tumors or the appearance of metastases. Preoperative excretory urography was performed to exclude cases with evidence of upper urinary tract disease.

All available hematoxylin and eosin stained slides including primary tumors as well as their recurrences were re-evaluated by two dedicated pathologists without knowledge of the clinical status, and were diagnosed and graded in accordance with the most recent World Health Organization grading system. Pathologic staging was determined in accordance with the TNM, 2010 revision.

Tissue-microarray

One hundred and two bladder carcinoma specimens were collected in a tissue-microarray (TMA) using a precision instrument (Tissue arrayer-Beecher Instruments, Silver Spring, MD). Representative tumor areas were identified and encircled on H&E-stained slides from each block. Two 0.6-mm tissue cylinders corresponding to the selected areas were removed from each paraffin donor block and placed side by side in an empty recipient

paraffin block measuring 27 x 20 mm. Hematoxylin and eosin stained slides of the TMA allowed to verify the adequacy of the samples.

Fluorescent In situ Hybridization (FISH)

A specific CCND3 locus probe (PAC clone RP5-973N23) labeled with Spectrum red-dUTP (red signal) (Vysis, Downers Grove, IL) was made. Briefly, isolated PAC DNA was labeled with Spectrum red-dUTP (Vysis) by nick translation (Vysis) and purified after adding 10 µg of COT-1 (Invitrogen, Carlsbad, CA, USA). A commercially available (CEP 6) probe (Vysis) labeled with spectrum green (green signal) was also used. To check the specificity of the home made probe, co-hybridizations using both commercial CEP6 and *CCND3* specific probes were performed over metaphases of peripheral blood cells.

FISH analysis was done on 2 µm-thick sections obtained from the tissue microarray. Negative controls were samples of unrelated tumors and adjacent normal mucosa or normal tissues of unrelated organs from 20 additional patients. A volume of 10 µl of the diluted probes was applied to the slides. The slide was covered with a glass cover slip and sealed with rubbercement (Royal Talens Apeldoorn, Holland). Using a Hybrite machine (Vysis) denaturation was 75°C for 5 min and hybridization was at 37°C for at least 16 h. After removing the cover slips post-hybridization washing was done at 46ºC in 2XSSC, 50% formamide for 5 min and stained with DAPI (6-diamidino-2-phenylindole) and mounted with Vectashield H-1000 medium (Vector). Digital images were obtained using a Zeiss Axioplan2 epifluorescence microscope (Carl Zeiss Oberkochen, Germany) equipped with a digital camera **(**ORCA-ER-1394**,** Hamamatsu Photonics KK, Hamamatsu, Japan). In all cases, one hundred nuclei were counted for each punch. The number of red signals (*CCND3*) and green signals (CEP6 centromeric) were counted for each nucleus and the average was recorded. We established a ratio red signal/green signal of 2 or greater as an evidence of *CCND3* gene amplification (16).

Immunohistochemistry

Immunohistochemical analysis was done on 4-µm-thick sections obtained from the tissue microarray. Then, dewaxed sections were rehydrated through phosphate buffered saline. For antigen retrieval, the sections were boiled in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase was blocked by incubating the slides for 30 min with 3% hydrogen peroxide in methanol. Sections were then incubated with anti-Cyclin D3 (clone DCS-22, Dako (Glostrup, Denmark, 1:25 dilution, 60 min) primary antibody at room temperature. Immunohistochemical stains were performed using a sensitive polymer-based system (EnVision system, Dako, Glostrup, Denmark) for 30 min at room temperature. Diaminobenzidine solution served as a chromogen. Sections were counterstained with hematoxylin and mounted using standard procedures. Positive (breast carcinoma) and negative controls (substituting the primary antibody with distilled water) were included.

Cyclin D3 immunohistochemistry was measured by using random fields delineated by a 1 cm^2 graded ocular grid attached to the eyepiece of the microscope (Nikon Labophot, Tokyo, Japan). The regions were chosen inside high immunoreactive areas examined under high power (×400) view and a mean of 300 cells per case were counted. A labeling index, defined as the percentage of positive nuclei, was established with tumors stratified following the low vs. high expression scheme <25% *vs* ≥25% (10).

Statistical analysis

Bivariate and multivariate analysis using contingency tables were tested for significance using *Chi*-square analysis and comparison of the means was carried out using the Student *t*-test. The end point of the study was recurrence-free and progression-free survival. Univariate survival analysis was conducted using the Kaplan-Meier method, and differences among groups were tested for significance using the Log-rank test. Significant parameters in the univariate analysis entered a multivariate analysis of independent prognostic factors using Cox proportional hazard regression analysis, and the relative risk with 95% confidence interval was calculated. All the statistical analyses were performed using the SPSS 15.0 for Windows Software (SPSS Inc, Chicago, IL). A *p* value of less than or equal to 0.05 was considered indicative of a statistically significant difference.

RESULTS

Table I shows patients' characteristics. A total of 102 patients (92 men) with bladder carcinoma were the study cohort. Their mean age was 71.08 (±SD, 11.17) years.

Most patients had high grade tumors (58.8%) with size < than 3 cm (66.7), and were multiple (58.8%). Pathologic stage was pTa (49%), pT1 (29.4%) or ≥pT2 (21.6), and 7.8% of patients had concurrent CIS. On follow up 47.5% of tumors recurred (mean±SD, 27.30±12.52, range 4-41 months and 23.5% progressed in stage (mean±SD,30.92±12.83, range 1-41 months). *CCND3* gene amplification seen by FISH as multiple scattered or grouped red signals was present in 31.4% of cases and was

Table I. *Clinico-pathologic features of bladder cancer according to CCND3 gene status.*

Variable	Overall N=102 (%)	CCND3 amplified N=32 (%)	CCND3 not amplified N=70 (%)	*P-value
Gender				0.662
Male	92 (90.2)	28 (87.5)	64 (91.4)	
Female	10 (9.8)	4 (12.5)	6 (8.6)	
§Age (y)	71.08±11.17 (45-92)	71.31±10.70 (54-85)	70.97±11.54 (45-92)	**0.921
Grade				0.330
LG	42 (41.2)	10 (31.3)	32 (45.7)	
HG	60 (58.8)	22 (68.8)	38 (54.3)	
pTcategory				0.171
Ta	50 (49)	12(37.5)	38 (54.3)	
T1	30 (29.4)	8 (25)	22 (31.4)	
≥T2	22 (21.6)	12 (37.5)	10 (14.3)	
CIS				0.775
Present	8 (7.8)	2 (6.3)	6(8.6)	
Absent	94 (92.2)	30 (93.8)	64 (91.4)	
Tumor size				0.670
≤ 3 cm	68 (66.7)	20 (62.5)	48 (68.6)	
> 3 cm	34 (33.3)	12 (37.5)	22 (31.4)	
Tumor number				0.718
Single	42 (41.2)	12 (37.5)	30 (42.9)	
Multiple	60 (58.8)	20 (62.5)	40 (57.1)	
¥Tumor recurrence				0.361
Yes	38(47.5)	12 (60)	26 (43.3)	
No	42(52.5)	8 (40)	34 (56.7)	
§Time to recurrence (months)	27.30±12.52 (4-41)	27.20±12.14 (5-41)	27.33±12.86 (4-41)	**0.977
Tumor progression				**0.021**
Yes	24 (23.5)	14(43.8)	10 (14.3)	
No	78 (76.5)	18 (56.3)	60 (85.7)	
§Time to progression (months)	30.92±12.83 (1-41)	25.75±15.25 (1-41)	33.29±11.00 (4-41)	****0.050**
§Cyclin D3 IHC status	38.86±30.46 (6-120)	76.69±27.51 (29-120)	21.57±7.02 (6-32)	****<0.0001**

** Chi-square analysis; ** Student t test; SD: standard deviation of the mean; LG: low grade; HG: high grade; IHC: immunohistochemistry: (y) age in years; §: mean±SD, range; ¥ tumor recurrence based on pTa/pT1 cases, N=80.*

associated to tumor progression (p=0.021), lower time to progression (p=0.050) and higher Cyclin D3 labeling index by immunohistochemistry (p<0.0001) (Table I, Fig. 1).

The univariate survival analysis showed that *CCND3* gene amplification was related to progression-free (p=0.020) but not to recurrence-free survival (p=0.558) (Tables II, III and Fig. 2). Cox's regression analysis selected concurrent CIS as independent predictor of recurrence-free survival (p=0.015, RR 7.108, CI1.458-34.653); and *CCND3* amplification (p=0.030, RR3.561, CI1.128-11.236), and pT stage (p<0.0001, RR 5.834, CI 2.364-14.395) as independent predictors of progression-free survival (Table IV).

DISCUSSION

Urothelial carcinoma of the urinary bladder is a distinct entity which frequently shows an unpredictable

Table II. *Univariate survival analysis according to recurrence-free survival (pTa-pT1 cases).*

Variable	Overall N=80	Recurrence-free (%)	Log-rank	*P*-value
Gender			0.989	0.320
Male	72	40 (55.6)		
Female	8	2 (25)		
Grade			0.000	0.998
LG	42	22 (52.4)		
HG	38	20 (52.6)		
pTstage			0.008	0.931
Ta	50	26 (52)		
T1	30	16 (53.3)		
CIS			8.093	**0.004**
Present	4	0 (0)		
Absent	76	42 (55.3)		
Tumor size			1.535	0.215
≤ 3 cm	56	34 (60.7)		
> 3 cm	24	8 (33.3)		
Tumor			1.366	0.243
Single	34	20 (58.8)		
Multiple	46	22 (47.8)		
CCND3 gene status			0.342	0.558
amplified	20	8 (40)		
not amplified	60	34 (56.7)		

CIS: Associated Urothelial Carcinoma In situ; LG: low grade; HG: high grade.

course (1-5). The untreated natural history indicates 30-50% stage progression rate and an even higher recurrence rate (1-5). Current BCG protocols in high grade tumors have improved substantially the prognosis of these patients, but in non-responding patients the risk of tumor progression is high.

Clinical factors are of limited help as predictors of aggressiveness in selected cases (1-5). Some recent reports, however, clearly indicate the value of p53, and Shariat et al (19) showed that a combined p53/p21Waf1 expression was associated with progression and cancer-specific survival. Hopman et al (20) also found an association between *TP53* mutation, loss of chromosome 9 and 9p21, and invasive bladder cancer in CIS patients. Loss of E-cadherin expression was reported as another risk factor for progression and disease-specific survival in patients with high grade urothelial carcinoma (21). Likewise, other molecular markers do not seem powerful enough to predict response to therapy or progression in daily practice (6, 7). Therefore, studies on newer molecular markers to define patterns of aggressiveness in bladder cancer are needed.

Cyclin D family, mainly Cyclin D1 and Cyclin D3, form complexes with cyclin dependent kinases 4 and 6 and thus promote phosphorylation and inactivation of the retinoblastoma protein releasing the promoter factor E2F-1 (13). This, in turns activates genes involved in DNA synthesis, thereby mediating the progression of cells from G1-to-S phase of cell cycle. Cyclin D3 expression in urothelial bladder cancer has received recent interest with some reports showing high cyclin D3 expression by immunohistochemistry to be an independent predictor of patients' survival

Table III. *Univariate survival analysis according to progression-free survival.*

Variable	Overall N=102	Progression-free (%)	Log-rank	*P*-value
Gender			0.001	0.973
Male	92	70 (76.1)		
Female	10	8 (80)		
Grade			10.261	**0.001**
LG	42	42 (100)		
HG	60	36 (60)		
pTcategory			23.679	**<0.0001**
Ta	50	50 (100)		
T1	30	20 (66.7)		
≥T2	22	8 (36.8)		
CIS			2.070	0.150
Yes	8	4 (50)		
No	94	74 (78.7)		
Tumor size			0.000	1.000
≤ 3 cm	68	52 (76.5)		
> 3 cm	34	26 (76.5)		
Tumor number			0.337	0.561
Single	42	34 (81)		
Multiple	60	44 (73.3)		
CCND3 **gene status**			5.414	**0.020**
Amplified	32	18 (56.3)		
Not amplified	70	60 (85.7)		

CIS: Associated Urothelial Carcinoma In situ; LG: low grade; HG: high grade.

Table IV. *Cox multivariate analysis showing independent variables related to recurrence and progression-free survival in patients with bladder cancer.*

Variable	RR	CI 95%	*P*-value
Recurrencen-free survival			
CIS	7.108	1.458-34.653	0.015
Progresión-free survival			
pT category	5.834	2.364-14.395	<0.0001
CCND3 gene status	3.561	1.128-11.236	0.030

CIS: Associated Urothelial Carcinoma In situ. CI: Confidence interval. RR: Risk ratio.

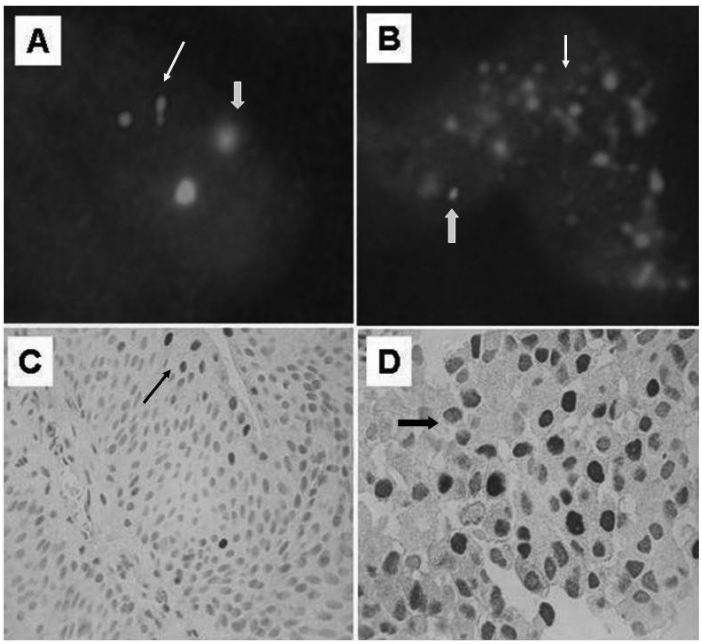

Fig. 1. *CCND3 gene normal expression (not amplified) showing two red signals (thin arrow) and two centromeric green signals (thick arrow) on chromosome 6 (normal expression) in bladder cancer (case 15) (**A**) as compared with CCND3gene amplification characterized by multiple red signals (thin arrow) in contrast to two centromeric green signals (thick arrow) on chromosome 6 (normal expression) in bladder cancer (case 26) (**B**) (**A** and **B**, FISH: fluorescence in situ hybridization). Low Cyclin D3 labeling index in case 15 showed not amplified (thin arrow) CCND3 gene by FISH (**C**) and high Cyclin D3 labeling index (thick arrow) in case 26 that showed amplified CCND3 gene by FISH(D) (**C** and **D**, anti-Cyclin D3 Immunohistochemistry).*

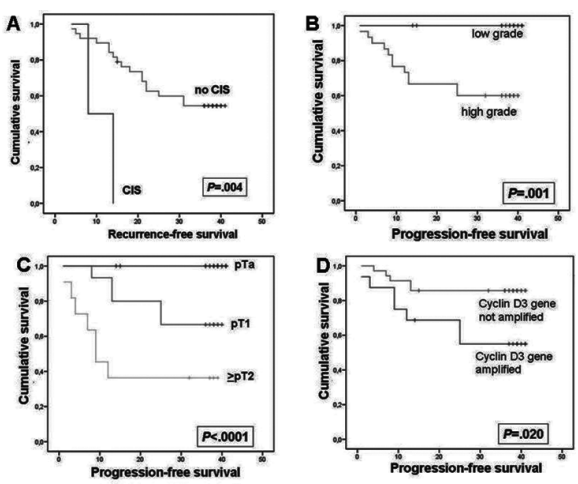

Fig. 2. *Kaplan–Meier plots illustrating the association between urothelial carcinoma in situ (CIS) and recurrence-free survival (months) (**A**), and histological grade (**B**), pT stage (**C**) and CCND3 gene status by FISH (**D**) associated to progression-free survival (months) in bladder cancer.*

(10, 11, 15, 17).

In the present study, *CCND3* gene status has been found to be independent predictor of progression-free survival. This is an original finding not previously reported. We found amplification and up-regulation of *CCND3* gene to be present in about one third of urothelial bladder cancer cases and in about two thirds of high grade carcinomas. This, is in agreement with our previous work showing cyclin D3 protein up-regulation in bladder carcinoma mainly in high grade cancers, (10, 11) an observation recently confirmed by Levidou et al. (15), and, also agrees with the finding of *CCND3* gene amplification by FISH in secondary urothelial carcinoma in situ but not in primary CIS cases; CIS is considered a high grade urothelial disease (16). Moreover, a recent study based on quantitative gene expression profiles showed that *CCND3* gene was a significant predictor or recurrence and progression in high grade bladder

cancer, thus supporting our findings on *CCND3* gene amplification by FISH in bladder cancer (22). Our observation of a strong association of *CCND3* gene amplification with progression-free survival could indicate that there is an important deregulation of *CCND3* gene along with protein overexpression in high grade bladder cancer. Moreover, it seems that cyclin D3 alteration is a potentially relevant finding since the use of cell cycle regulatory proteins as therapeutic targets of their modulators, represents an active field of research (6, 13-16, 23-25). Available modulators of Cyclin D3 expression such Interferon-a and flavopiridol, that can induce cell cycle arrest by directly inhibiting cyclin dependent kinases and by down-regulation of Cyclin D3, might represent a future alternative to other conventional therapeutic approaches in a subset of bladder cancer patients at higher risk of aggressive behavior. In fact, recent studies show that cyclin D3 might be a potential biomarker of response to targeted therapy in neuroblastoma and lung cancer; a finding that awaits confirmation in bladder cancer patients (6, 13-16, 23-25). Recent reports support the role of cyclin D3 in different pathways known to be altered in bladder cancer, i.e. cyclin D3 is also a downstream STAT3 target such as Mcl-1, Bcl-2, and Bcl-xL, all of which are implicated in apoptosis and the cell cycle. (6, 13-16, 23-25) There was greater expression of the PI3K-AKT-cyclin D3 molecular pathway downstream to the activation of pPDGF-betaR in neoplastic compared with normal tissue in bovine bladder cancer (17). Cyclin D3 might be a microRNA (MiR-138) target in hepatocellular carcinoma (26). This new information suggests a role of cyclin D3 in bladder cancer biology and pathology.

Our study also highlights that *CCND3* gene amplification is associated to higher cyclin D3 protein labeling index, suggesting that over-expression of Cyclin D3 protein, as detected by immunohistochemistry, is mediated by gene amplification. Observed differences between *CCND3* gene and Cyclin D3 protein expression concerning significance in predicting bladder cancer progression, might be related to the quantification method of the immunohistochemistry, which is far to be as precise as the quantification of FISH color signals.

In conclusion, we have shown the apparent role of *CCND3* gene amplification and up-regulation as a biomarker which helps to select a subset of tumors at higher risk of developing invasive disease. This warrants further prospective studies on the expression of this cell cycle regulatory gene and protein in bladder cancer patients.

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REFERENCES

1. Lopez-Beltran A. Bladder cancer: clinical and pathological profile Scand J Urol Nephrol Suppl 2008; 218:95-9.
2. Eble JN, Sauter G, Epstein JI, Sesterhenn IAE. World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs. IARC Press Lyon 2004.
3. Lopez-Beltran A, Montironi R. Non-Invasive Urothelial Neoplasms: According to the most recent WHO classification. Eur Urol 2004; 46:170-6.
4. Mazzucchelli R, Cheng L, Lopez-Beltran A, Scarpelli M, Montironi R. Classification and grading of noninvasive and invasive neoplasms of the urothelium. Anal Quant Cytol Hitol 2012; 34:111-9.
5. Cheng L, Zhang S, Davidson DD, et al. Molecular determinants of tumor recurrence in the urinary bladder. Future Oncol. 2009; 5:843-57.
6. Gonzalez-Campora R, Davalos-Casanova G, Beato-Moreno A, Lopez-Beltran A. BCL-2, TP53 and BAX protein expression in superficial urothelial bladder carcinoma, Cancer Lett 2007; 250:292-9.
7. Zlotta AR, Noel JC, Fayt I. Correlation and prognostic significance of p53, p21WAF1/CIP1 and Ki-67 expression in patients with superficial bladder tumors treated with bacillus Calmette-Guerin intravesical therapy J Urol 1999; 161:792-8
8. Lopez-Beltran A, Cheng L, Mazzucchelli R, Blanca

A, Montironi R. Morphological and Molecular Profiles and Pathways in Bladder Neoplasms. Anticancer Res 2008; 28:2893-0

9. Schrier BP, Vriesema JL, Witjes JA, Kiemeney LA, Schalken JA. The predictive value of p53, p27(kip1), and alpha-catenin for progression in superficial bladder carcinoma. Eur Urol 2006; 50:76-2
10. Lopez-Beltran A, Requena MJ, RJ Luque, Alvarez J, Montironi R. Cyclin D3 expression in primary Ta/T1 bladder cancer J Pathol 2006; 209:106-3.
11. Lopez-Beltran A, Luque RJ, Alvarez-Kindelan J, Montironi R. Prognostic Factors in StageT1Grade 3 Bladder Cancer Survival: The Role of G1+SModulators (p53, p21Waf1, p27kip1, Cyclin D1, and Cyclin D3) and Proliferation Index (ki67-MIB1). Eur Urol 2004; 45:606-2.
12. Bartkova J, Zemanova M, Bartek J. Abundance and subcellular localization of cyclin D3 in human tumors Int J Cancer 1996; 65:323-7.
13. Pruneri G, Pignataro L, Valentini S. Cyclin D3 Immunoreactivity Is an Independent Predictor of Survival in Laryngeal Squamous Cell Carcinoma. Clin Cancer Res 2005; 11:242-8.
14. Petty WJ, Voelzke WR, Urbanic JJ, et al. High cyclin D3 expression confers erlotinib resistance in aerodigestive tract cancer. Lung Cancer 2011; 74:384-1.
15. Levidou G, Saetta AA, Karlou M et al. D-type cyclins in superficial and muscle-invasive bladder urothelial carcinoma: correlation with clinicopathological data and prognostic significance. J Cancer Res Clin Oncol 2010; 136:1563-71.
16. Lopez-Beltran A, Ordóñez JL, Otero AP, et al. Cyclin D3 gene amplification in bladder carcinoma in situ. Virchows Arch 2010; 457:555-61.
17. Corteggio A, Urraro C, Roperto S, Roperto F, Borzacchiello G. Phosphatidylinositol-3-kinase-AKT pathway, phospho-JUN and phospho-JNK expression in spontaneously arising bovine urinary bladder tumours. J Comp Pathol 2010; 143:173-8.
18. Yang F, Jove V, Buettner R, et al. Sorafenib inhibits endogenous and IL-6/S1P induced JAK2-STAT3 signaling in human neuroblastoma, associated with growth suppression and apoptosis. Cancer Biol Ther 2012; 13:534-41.
19. Shariat SF, Kim J, Raptidis G, Ayala GE, Lerner SP. Association of p53 and p21 expression with clinical outcome in patients with carcinoma in situ of the urinary bladder. Urology 2003; 61:1140-5.
20. Hopman AH, Kamps MA, Speel EJ, Schapers RF, Sauter G, Ramaekers FC. Identification of chromosome 9 alterations and p53 accumulation in isolated carcinoma in situ of the urinary bladder versus carcinoma in situ associated with carcinoma. Am J Pathol 2002; 161:1119-25.
21. Shariat SF, Pahlavan S, Baseman AG. E-cadherin expression predicts clinical outcome in carcinoma in situ of the urinary bladder. Urology 2001; 57:60-5.
22. Birkhahn M, Mitra AP, Williams AJ. Predicting Recurrence and Progression of Noninvasive Papillary Bladder Cancer at Initial Presentation Based on Quantitative Gene Expression Profiles Eur Urol 2010; 57:12-20.
23. Song YA, Park YL, Kim KY, et al. RON is associated with tumor progression via the inhibition of apoptosis and cell cycle arrest in human gastric cancer. Pathol Int 2012; 62:127-6.
24. Wang X, Sistrunk C, Miliani de Marval PL, Kim Y, Rodriguez-Puebla ML. Combined effect of cyclin D3 expression and abrogation of cyclin D1 prevent mouse skin tumor development. Cell Cycle 2012; 11:335-42.
25. Zhang Q, Sakamoto K, Liu C, et al. Cyclin D3 compensates for the loss of cyclin D1 during ErbB2-induced mammary tumor initiation and progression. Cancer Res 2011; 71:7513-4.
26. Wang W, Zhao LJ, Tan YX, Ren H, Qi ZT. MiR-138 induces cell cycle arrest by targeting cyclin D3 in hepatocellular carcinoma. Carcinogenesis. 2012; 33:1113-20.