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ORIGINAL ARTICLE

mRNA expression of G_1-phase cell cycle regulatory molecules in bladder cancer

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Summary

Purpose: *Cell cycle regulation, which is important for normal cellular proliferation, is controlled by a complex network of intracellular proteins, with cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs) playing a central role. This equilibrium is interrupted in cancer cells, resulting in uncontrolled cellular proliferation.*

Methods: *In the present study we examined, by means of semi-quantitative RT-PCR, the expression of G_1-phase cell cycle regulators MDM2, E2F1, Cyclin D1 (CCND1), CDK4, $p19^{INK4D}$, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in a series of 32 bladder cancer specimens paired with adjacent normal tissues.*

Results: *Cyclin D1 was overexpressed in 10/32 (31.2%) and downregulated in 8/32 (25.0%) bladder cancer specimens. Additionally, p21 was overexpressed in 9/32 (28.1%) and downregulated in 10/32 (31.3%) cancer samples. On the contrary, MDM2, E2F1, CDK4, p19 and p27 expression was normal in the majority of malignant specimens. Further statistical analysis revealed significant associations between increased p21 levels and bladder cancer patients with no exposure to chemicals (p=0.048), as well as with patients with no artificial sweetener intake (p=0.012), and between increased Cyclin D1 levels and study subjects with no artificial sweetener intake (p=0.012).*

Conclusion: *Based on these results, we conclude that Cyclin D1 and p21 mRNA deregulation seems to be an important event in bladder carcinogenesis. However, further studies are needed, in order to determine whether these two cell cycle regulators can be used as markers for the early detection of bladder cancer and to monitor its progression and recurrence.*

Key words: bladder cancer, CDKI, CDK, Cyclin D1, E2F1, MDM2, RT-PCR

Introduction

Bladder cancer is the second most common cancer of the genitourinary tract. It is the 4th most common malignancy in men and the 12th most common in women [1]. Environmental factors that have been associated with this disease include occupational exposure to chemicals, cigarette smoking, coffee drinking, artificial sweetener intake and bacterial, parasitic or viral infections [2].

Disruption of normal cell cycle regulation is a common event in malignant cells that leads to uncontrolled cell proliferation during tumor development [3]. Orderly progression through the different phases of the cell cycle is controlled by cyclins, CDKs and their inhibitors (CDKIs). CDKIs are negative regulators of the cell cycle and can also act as tumor-suppressor genes. Their loss of expression plays an important role in the development of human tumors [4].

Cyclin D1 (CCND1) is a known oncogene located at chromosome 11q13 [5]. It plays an important role in the regulation of the G1→S phase transition of the cell cycle [6]. Cyclin D1 has been found to be overexpressed in several neoplasias, including bladder cancer [7], but its expression was higher in superficial rather than in muscle-invasive tumors [8]. CDK4, which binds to Cyclin D1 to form the pRb kinase [9], has also been found to be expressed in bladder cancer, but was downregulated in more advanced neoplasias [10].

$p19^{INK4D}$, a member of the INK4 CDKI family [11], is located at 19p13 and encodes a protein that interacts physically with CDK4 and CDK6 and inhibits their cyclin D-dependent kinase activities [12]. The p19 locus is frequently deleted in bladder squamous cell carcinomas (SCC), but not in transitional cell carcinomas (TCC) [13].

The CIP/KIP CDKI family includes $p21^{WAF/CIP1}$

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and p27^{KIP1}, which suppress cell cycle progression through inhibition of diverse Cyclin-CDK complexes [14,15]. p21 activation is an early event in bladder carcinogenesis, while its downregulation is associated with tumor invasiveness [16]. Additionally, p27 expression is reduced in a variety of human malignancies, including bladder cancer and is associated with tumor progression [17].

The MDM2 gene is located on chromosome 12q13 -14 and encodes a nuclear protein capable of forming complexes with both wild-type and mutant p53 protein. MDM2 plays an important role in controlling p53 activity. It binds to p53 protein and increases its susceptibility to proteolysis by the proteosome [18]. MDM2 expression has been found to be downregulated in advanced high-grade, high-stage bladder tumors when compared to superficial low-grade, low-stage carcinomas [19].

The E2F1 transcription factor is a downstream effector of the tumor onco-suppressor gene pRB [20] and has a pivotal role in controlling cell cycle progression, since it determines the timely expression of many genes required for entry into and progression through the S phase of the cell cycle [21]. Bladder cancer patients with low E2F1 expression exhibit an increased risk of progression to metastatic disease [22].

In the present study we measured, using a semi-quantitative RT-PCR assay, the mRNA expression of $G_1 \rightarrow S$ cell cycle regulatory molecules MDM2, E2F1, Cyclin D1, CDK4, p19^{INK4D}, p21$^{WAF/CIP1}$ and p27^{KIP1}, in bladder cancer and adjacent normal tissue specimens, and examined their association with patient clinicopathological parameters.

Table 1. Clinicopathological characteristics of the study subjects

Characteristics	*Bladder cancer patients (%)*
Cases, n	32
Age, years (mean±SD)	71.3±7.2
Gender	
Male	32 (100.0)
Female	0 (0.0)
Tumor stage	
Ta	9 (28.1)
T1	15 (46.9)
T2-T4	8 (25.0)
Tumor grade	
I	5 (15.6)
II	13 (40.6)
III	14 (43.8)
Exposure to chemicals	
Yes	10 (31.2)
No	22 (68.8)
Smoking status	
Current smokers	18 (56.2)
Ex-smokers	3 (9.4)
Non-smokers	11 (34.4)
Artificial sweeteners	
Yes	21 (65.6)
No	11 (34.4)
Bladder infections	
Yes	10 (31.2)
No	22 (68.8)
Bladder/kidney stones	
Yes	4 (12.5)
No	28 (87.5)
Systemic diseases	
Yes	13 (40.6)
No	19 (59.4)
Cancer family history	
Yes	11 (34.4)
No	21 (65.6)

Methods

Study subjects

Malignant and adjacent normal tissue specimens were collected from 32 male patients that underwent surgical treatment for bladder TCC at the Department of Urology, University Hospital of Heraklion, Crete, Greece. After surgical removal, samples were snap-frozen in liquid nitrogen and stored at –80° C until used. The median patient age at diagnosis was 71.5 years (range 54-85). Tumor stage and grade were evaluated by histological examination using hematoxylin & eosin-stained slides, according to the UICC TNM classification system and the 1973 WHO grading system, respectively. The clinicopathological characteristics of the patients are listed in Table 1. The ethics committees of the University of Crete and the University Hospital of Heraklion approved this study, and written informed consent was obtained from all participants.

RNA extraction

A 100-mg sample of each tissue specimen was homogenized in 1 mL of TRIzol® reagent (Invitrogen, Carlsbad, CA) using a power homogenizer, followed by the addition of chloroform and centrifugation. Total RNA was precipitated from the supernatant with isopropanol, washed with 75% ethanol and resuspended in 50 μL of diethylpyrocarbonate (DEPC)-treated water. RNA concentration and purity were calculated after measuring the 260-nm absorbance and 260/280-nm absorbance ratio, respectively, on a UV spectrophotometer.

cDNA preparation

cDNA was synthesized by reverse transcription (RT) with the Thermoscript™ RT kit (Invitrogen), using

random hexamers as amplification primers. In detail, 2 μg of total RNA, 50 ng of random hexamers and 1 mM deoxynucleotide triphosphates (dNTPs) were heated at 65° C for 5 min, in order to remove RNA secondary structures, and placed on ice until the addition of cDNA synthesis mix, which contained 1×·cDNA synthesis buffer (50 mM Tris-acetate, pH 8.4, 75 mM potassium acetate and 8 mM magnesium acetate), 5 mM dithiothreitol (DTT), 40 units RNaseOut™ and 15 units Thermoscript™ reverse transcriptase. The final mix (volume 20 μL) was incubated for 10 min at 25° C for primer extension, and cDNA synthesis was conducted at 55°C for 50 min. The reaction was terminated by heating at 85° C for 5 min. In order to remove the RNA template, cDNA was incubated at 37° C for 20 min with 2 units of *Escherichia coli* RNaseH, and stored at –20° C until use.

Semi-quantitative polymerase chain reaction (RT-PCR)

mRNA expression of cell cycle regulators MDM2, E2F1, CCND1, CDK4, p19, p21 and p27 was measured using a semi-quantitative PCR assay. The housekeeping gene, β2-microglobulin (β2M), was used as internal control, in order to normalize the mRNA expression levels of the studied genes. The mRNA-specific primers, which were designed with the Lasergene® software (DNASTAR, Madison, WI) spanning at least one intron with an average length >800 bp, are listed in Table 2. Their specificity was verified with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). cDNA (1 μl) from normal and malignant samples was amplified in a PCR reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 300 nM of each primer pair and 0.5 units Platinum Taq DNA Polymerase (Invitrogen), in a final volume of 20 μl. After initial denaturation at 95° C for 3 min, samples were subjected to 33 cycles of amplification (which corresponds to the linear part of the amplification curve), comprised of denaturation at 95° C for 30 sec, annealing at the appropriate temperature (51-58° C) for 30 sec and elongation at 72° C for 45 sec. PCR experiments were conducted on an PTC-200 PCR thermal cycler (MJ Research Inc., Waltham, MA). In each PCR reaction two negative controls were included, one with no cDNA template and one with no reverse transcription treatment. All PCR experiments were conducted in triplicates.

Analysis of the expression data

PCR products were analyzed on 8.5% polyacrylamide gels (acrylamide/bis-acrylamide ratio 29:1) and silver stained. Gels were scanned on an Agfa SnapScan 1212u scanner (Agfa-Gevaert Group, Mortsel, Belgium). The integrated density of each band was used as a quantitative parameter and was calculated using Photoshop CS2 image analysis software (Adobe Systems Inc., San Jose, CA). To normalize the mRNA expression of each gene studied, its value was divided by the β2M mRNA value. The normalized value of each tumor sample was divided by the normalized value of its adjacent normal sample. The result of this division provided the relative expression of the tumor specimen in relation to its normal counterpart. A 2-fold increased (a value ≥2) or decreased (a value ≤0.5) expression was considered biologically significant (overexpression or downregulation, respectively).

Table 2. Primer pair oligonucleotide sequences, optimum annealing temperatures and PCR products sizes

Primer pair	*Oligonucleotide sequence (5'-3')*	*Temperature (° C)*	*Amplicon size (bp)*
MDM2	TGAAGGTTTCTCTTGCTGAAGC AAGGTGGGAGTGATCAAAAGG	51	180
E2F1	GTGCAGATTGGAGGGTGG GATGGATATGAGATGGGAGAGG	58	154
CCND1	AAAGACAGTTTTTGGGTAATCTTTT CCGGAGCATTTTGATACCAG	54	126
CDK4	CTTCTGGACACTGAGAGGGC TGGGAGGGGAATGTCATTAA	54	110
p19	GATCTGGGGTCACCCTCTC CCAACACACCAAAAGGAGTG	54	207
p21	ATTCAGCATTGTGGGAGGAG TGGACTGTTTTCTCTCGGCT	57	131
p27	GGATAAGTGAAATGGATACTACATC AAAAAGAGGGGAAAACCTATTCTAC	58	228
β2M	AGCGTACTCCAAAGATTCAGGTT TACATGTCTCGATCCCACTTAACTAT	–	297

Statistical analysis

MDM2, E2F1, CCND1, CDK4, p19, p21 and p27 mRNA levels were first evaluated by the one-sample Kolmogorov-Smirnov goodness-of-fit test, in order to determine whether they followed a normal distribution pattern. Depending on the results, Pearson's or the non-parametric Spearman's rank test were used to examine their relationship pairwise and their association with continuous variables (age). Moreover, their association with categorical data (tumor stage and grade, exposure to chemicals such as fertilizers and pesticides, smoking status, artificial sweetener intake, bladder infections, bladder/kidney stones, systemic diseases and cancer family history) was examined using the Student's t-test (after an assessment of the equality of variances using Levene's test), or its non-parametric equivalents Mann-Whitney U and Kruskal-Wallis H tests. Additionally, the chi-square (x^2) test, replaced by Fisher's exact test when indicated by the analysis, was used to examine the cell cycle regulator expression status with the various clinicopathological parameters after stratification. Finally, Spearman's rank correlation was used to examine in a pair-wise manner the mRNA expression of the cell cycle molecules studied in both the normal and malignant tissue groups. Statistical analyses were 2-sided and performed with SPSS 11.5 (SPSS Inc., Chicago, IL). Statistical significance was set at the 95% level (p-value < 0.05).

Results

In the present study we evaluated the mRNA expression of MDM2, E2F1, CCND1, CDK4, p19, p21 and p27, in a total of 32 bladder cancer specimens and adjacent normal controls using a semi-quantitative RT-PCR method.

MDM2 and p27 mRNA expression was decreased in the tumor specimens compared to the normal samples (1.06±0.07 vs. 1.35±0.091.32, 2-tailed Mann-Whitney U test, p=0.017 and 0.98±0.06 vs. 1.24±0.07, 2-tailed Mann-Whitney U test, p=0.007, respectively) (Figure 1). However, the transcription levels of the other 5 genes tested did not differ statistically between the normal and tumor samples (Table 3).

Using the Spearman's rank test, we tested the co-expression pattern of the 7 studied genes in a pair-wise manner in both the cancer and adjacent normal bladder specimens. This test examines whether two molecules are upregulated or downregulated together (positive association), or whether when one is overexpressed the other has reduced expression (negative association) and *vice versa*.

Table 3. mRNA expression of G1-phase cell cycle regulators in malignant and adjacent normal bladder samples

Cell cycle regulator	*Tumor**	*Normal**	*p-value*†
MDM2	1.06±0.07	1.35±0.09	**0.017**
E2F1	1.10±0.07	1.29±0.08	0.13
CCND1	2.48±0.36	2.48±0.25	0.66
CDK4	1.06±0.06	1.03±0.04	0.60
p19	0.53±0.04	0.63±0.05	0.10
p21	3.31±0.51	3.24±0.46	0.92
p27	0.98±0.06	1.24±0.07	**0.007**

*Data are presented as mean ± SEM (standard error of the mean) after normalization with the expression of β2M.
†2-tailed Mann-Whitney U test. Bold entries indicate statistically significant results.

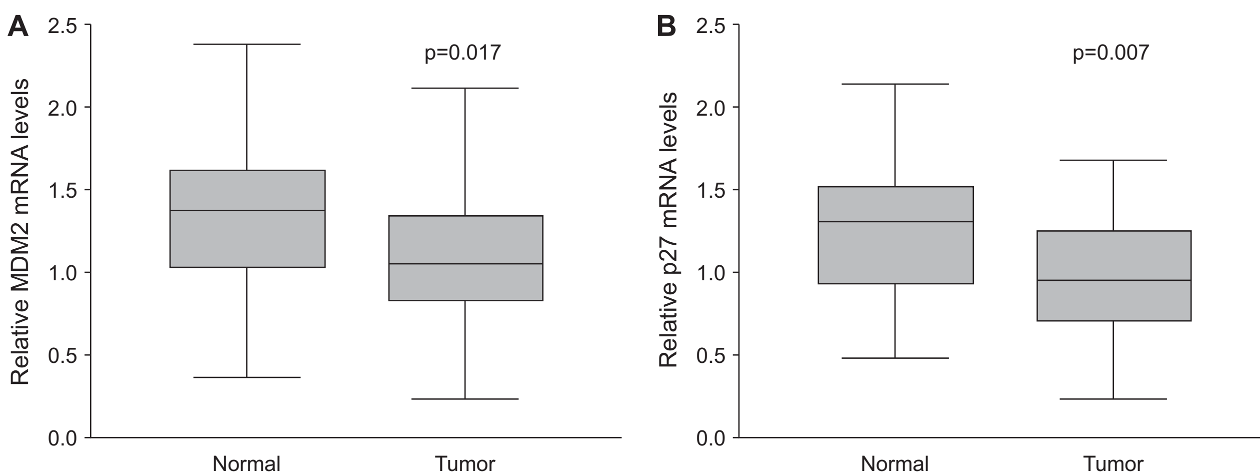

Figure 1. Box and whisker plots depicting statistically significant differences in the mRNA expression of the cell cycle regulatory molecules between normal and malignant bladder samples. **A:** MDM2 mRNA levels are lower in bladder cancer specimens compared to adjacent normal samples (1.06±0.07 vs. 1.35±0.091.32, p=0.017); **B:** p27 expression is decreased in bladder cancer specimens compared to adjacent normal samples (0.98±0.06 vs. 1.24±0.07, p=0.007). All values are presented as mean ± SEM. Statistical analysis was conducted using the 2-tailed Mann-Whitney U test.

Table 4. Analysis of the cell cycle regulator pair-wise co-expression

A. Normal bladder specimens

		MDM2	*E2F1*	*CCND1*	*CDK4*	*p19*	*p21*	*p27*
MDM2	CC	1.000						
	p-value							
E2F1	CC	0.026	1.000					
	p-value	0.887						
CCND1	CC	–0.158	–0.039	1.000				
	p-value	0.423	0.842					
CDK4	CC	0.475	–0.001	0.273	1.000			
	p-value	**0.006**[b]	0.994	0.160				
p19	CC	0.514	0.204	0.365	0.810	1.000		
	p-value	**0.003**[b]	0.264	0.056	**0.000**[b]			
p21	CC	0.245	0.077	0.424	0.758	0.727	1.000	
	p-value	0.200	0.692	**0.028**[a]	**0.000**[b]	**0.000**[b]		
p27	CC	–0.018	0.287	0.350	0.189	0.243	0.244	1.000
	p-value	0.923	0.112	0.068	0.299	0.180	0.203	

B. Malignant bladder samples

		MDM2	*E2F1*	*CCND1*	*CDK4*	*p19*	*p21*	*p27*
MDM2	CC	1.000						
	p-value							
E2F1	CC	0.424	1.000					
	p-value	**0.016**[a]						
CCND1	CC	0.185	0.338	1.000				
	p-value	0.345	0.079					
CDK4	CC	0.520	0.757	0.490	1.000			
	p-value	**0.002**[b]	**0.000**[b]	**0.008**[b]				
p19	CC	0.535	0.796	0.463	0.794	1.000		
	p-value	**0.002**[b]	**0.000**[b]	**0.013**[b]	**0.000**[b]			
p21	CC	0.328	0.608	0.583	0.644	0.625	1.000	
	p-value	0.076	**0.000**[b]	**0.001**[b]	**0.000**[b]	**0.000**[b]		
p27	CC	0.553	0.783	0.473	0.594	0.734	0.662	1.000
	p-value	**0.001**[b]	**0.000**[b]	**0.011**[a]	**0.000**[b]	**0.000**[b]	**0.000**[b]	

All statistical tests are 2-tailed. CC: Correlation coefficient. [a]Correlation is significant at the 0.05 level. [b]Correlation is significant at the 0.01 level.

The results of this analysis are shown in Table 4. In normal bladder (Table 4A), 6 positive cases of co-expression were found. In bladder cancer (Table 4B), 12 more cases of positive co-expression were observed. From the above results we deduce that the co-expression profile of the G_1-phase cell cycle regulatory molecules studied differs significantly between normal and malignant bladder.

Further analysis of the expression data revealed that Cyclin D1 was overexpressed in 10/32 (31.2%) and downregulated in 8/32 (25.0%) bladder cancer samples. Additionally, p21 was overexpressed in 9/32 (28.1%) and downregulated in 10/32 (31.3%) bladder cancer samples. In contrast, MDM2, E2F1, CDK4, p19 and p27 expression was normal in the majority of the malignant specimens (Table 5).

Statistical analysis of the transcription levels of the cell cycle regulators and the patient clinicopathological parameters revealed several significant associations (Figure 2). p21 mRNA levels were elevated in the bladder cancer patients with no exposure to chemicals vs. the patients with exposure to chemicals (1.88±0.67 vs.

Table 5. Relative mRNA expression of the cell cycle regulators in bladder cancer specimens

Cell cycle regulator	*Relative mRNA expression* ↑ *(%)*	– *(%)*	↓ *(%)*
MDM2	0/32 (0.0)	26/32 (81.2)	6/32 (18.8)
E2F1	2/32 (6.3)	25/32 (78.1)	5/32 (15.6)
CCND1	10/32 (31.2)	14/32 (43.8)	8/32 (25.0)
CDK4	1/32 (3.1)	29/32 (90.6)	2/32 (6.3)
p19	5/32 (15.6)	20/32 (62.5)	7/32 (21.9)
p21	9/32 (28.1)	13/32 (40.6)	10/32 (31.3)
p27	0/32 (0.0)	26/32 (81.2)	6/32 (18.8)

↑ overexpression; – normal expression; ↓ reduced expression

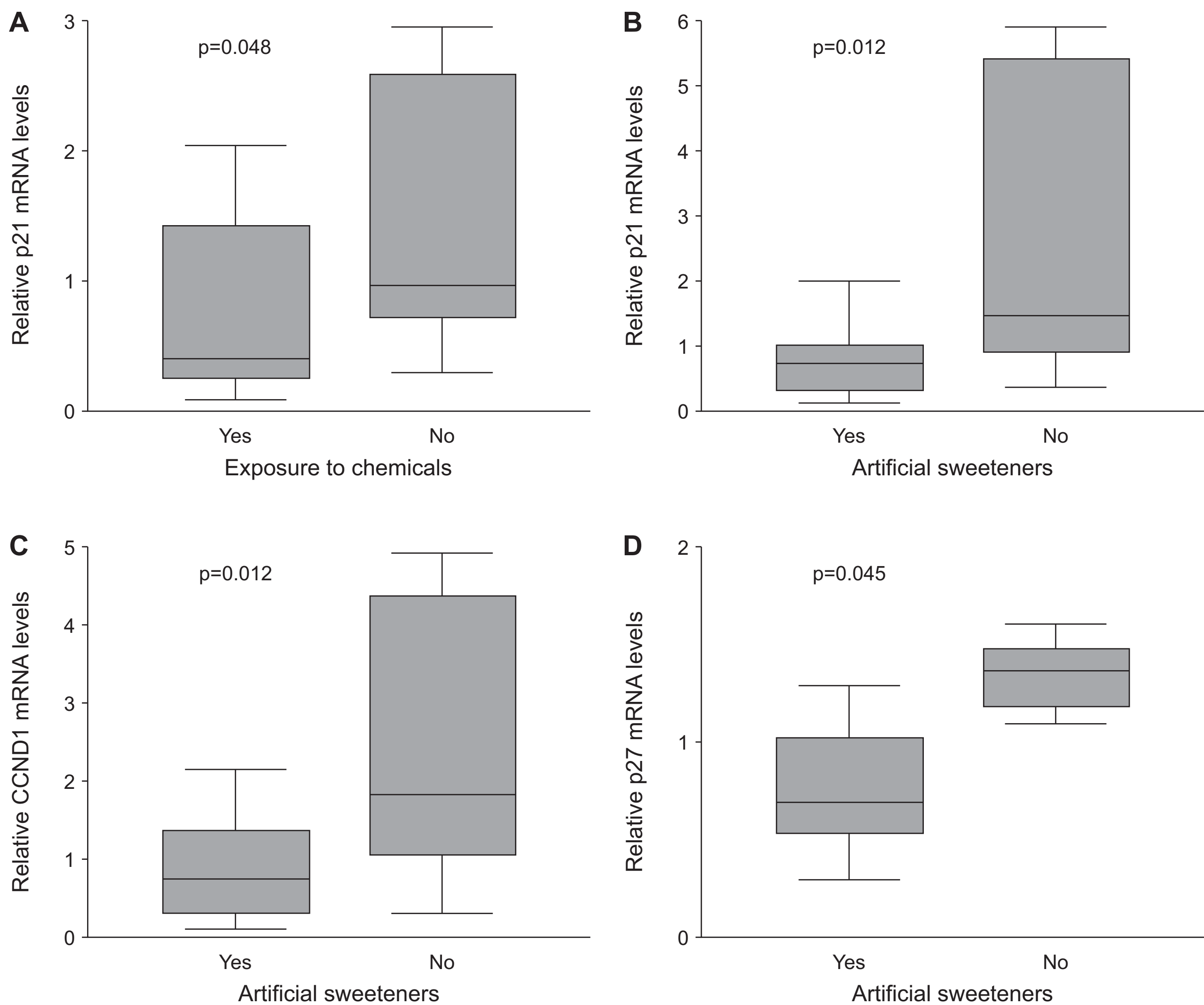

Figure 2. Box and whisker plots depicting statistically significant associations between the relative mRNA expression of the cell cycle regulators and various clinical parameters in the malignant bladder specimens. **A:** p21 mRNA levels are elevated in bladder cancer patients with no exposure to chemicals vs. patients with exposure to chemicals (1.88±0.67 vs. 0.91±0.33, p=0.048); **B:** p21 expression is increased in study subjects with no artificial sweetener intake vs. subjects with artificial sweeteners intake (3.55±2.24 vs. 0.94±0.20, p=0.012); **C:** CCND1 mRNA expression is higher in cancer patients with no artificial sweetener intake vs. patients with artificial sweetener intake (2.43±1.13 vs. 1.10±0.26, p=0.012); **D:** p27 mRNA levels are increased in bladder cancer subjects with no artificial sweetener intake vs. subjects with artificial sweetener intake (1.34±0.05 vs. 0.77±0.08, p=0.045). All values are presented as mean ± SEM. Statistical analysis was conducted using the 2-tailed Mann-Whitney U test.

0.91±0.33, 2-tailed Mann-Whitney U test, p=0.048); Notably, p21 expression was also increased in the study subjects with no artificial sweetener intake vs. subjects with artificial sweeteners intake (3.55±2.24 vs. 0.94±0.20, 2-tailed Mann-Whitney U test, p=0.012). Additionally, CCND1 mRNA expression was higher in the cancer patients with no artificial sweetener intake vs. the patients with artificial sweetener intake (2.43±1.13 vs. 1.10±0.26, 2-tailed Mann-Whitney U test, p=0.012). Finally, p27 mRNA levels were increased in the bladder cancer subjects with no artificial sweetener intake vs. the subjects with artificial sweetener intake (1.34±0.05 vs. 0.77±0.08, 2-tailed Mann-Whitney U test, p=0.045). All other associations tested were not statistically significant.

Discussion

Cell cycle deregulation is a common observation in malignantly transformed tissues leading to uncontrolled cell proliferation. In the present study, we evaluated the mRNA expression of some of the key regulatory molecules of the G1→S cell cycle phase (MDM2, E2F1, Cyclin D1, CDK4, $p19^{INK4D}$, $p21^{WAF/CIP1}$ and $p27^{KIP1}$) in a series of malignant and adjacent normal bladder tissues. The mRNA expression levels obtained were associated with the patient clinicopathological features, following co-expression analysis.

According to our results, the mean value of MDM2 mRNA expression in bladder cancer specimens was

lower than that observed in the adjacent normal bladder tissues (p=0.017). p53 and MDM2 form a feedback loop in which p53 positively regulates MDM2 transcription, and MDM2 negatively regulates p53 by promoting p53 ubiquitination and degradation [23]. The difference in MDM2 transcript levels between the malignant and normal bladder tissues suggests that this autoregulatory feedback machinery is probably disrupted in bladder cancer.

The transition from G1→S phase is controlled by two parallel pathways. One involves Cyclin E-CDK2 and is independent of pRb and E2F. The other one requires the phosphorylation of pRb by the Cyclin D-CDK4/6 complexes in order to generate free active E2F. The cellular requirement for pRb phosphorylation normally associated with the G1→S transition can be abolished when E2F1 is overexpressed [24]. However, our results demonstrated that E2F1 levels were normal in the majority of the bladder cancer specimens, suggesting that this regulatory mechanism is not disrupted in bladder carcinogenesis.

Cyclin D1 mRNA levels were deregulated in bladder cancer (overexpression in 31.2% and downregulation in 25.0% of malignant samples, respectively). These results are in concordance with other investigators [25]. Additionally, CCND1 expression was also affected by artificial sweetener intake. Overexpression of cyclins increases the concentration of active Cyclins/CDK complexes in the cell, thereby stimulating the G1→S transition of the cell cycle [26]. Overexpression of CDKs could therefore constitute an alternative mechanism promoting inappropriate cell proliferation. However, this did not occur in bladder cancer, since, according to our results, CDK4 mRNA levels did not exhibit any differences between the normal and malignant bladder samples.

It has been suggested that downregulation of CDK inhibitors plays an important role in bladder tumorigenesis. Loss of p21, which inhibits cell proliferation in either a p53-dependent or p53-independent manner, but does not mediate p53-induced apoptosis in urothelial cells [27], has been observed in both superficial and invasive bladder tumors. It has been associated with aggressive tumor behavior and is a predictor of tumor progression [28,29]. Our results partially agree with the above observations, since p21 was upregulated in 28.1% and downregulated in 31.3% of the bladder cancer cases, respectively. It is also noteworthy that p21 mRNA levels were affected by both exposure to chemicals and artificial sweetener intake. Reduced p27 expression has also been reported for numerous types of malignancies, including urothelial bladder cancer [30]. However, in our bladder cancer cases, p27 expression was normal, as was the expression of p19, indicating that these two CDKIs do not play an important role in bladder cancer development and progression.

mRNA co-expression analysis of all of the cell cycle regulatory molecules included in this study led to some very interesting observations. More and considerably stronger positive correlations were present in the neoplastic bladder tissue group when compared to the normal bladder tissue group. These findings indicate that the malignant transformation of the bladder is accompanied by many alterations in the transcriptional machinery of the urothelial cell.

In conclusion, our results provide evidence that the deregulation of Cyclin D1, and p21 mRNA expression is involved in the tumorigenic process of the urothelium, and that exposure to chemicals and artificial sweeteners also plays an important role in the neoplastic process. Additionally, the different co-expression patterns of the genes examined in this study suggest a transcriptional deregulation during the development and progression of bladder cancer. Further studies are required in order to elucidate the potential use of mRNA expression profiles of cell cycle regulatory molecules as biomarkers of development, progression and recurrence of bladder cancer.

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