

## ARTICLE

## Reduced E-cadherin and $\alpha$ -catenin Expressions Have No Prognostic Role in Bladder Carcinoma

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In various human cancers, dysfunction of the E-cadherin-catenin complex is associated with a decrease in cellular and tissue differentiation, and with higher invasive and metastatic potentials. The objective of this study was to investigate E-cadherin and  $\alpha$ -catenin expression in superficial noninvasive papillary TCC and invasive TCC, and correlate these results with pathological and clinical parameters. We have used immunohistochemistry to localize E-cadherin and  $\alpha$ -catenin in 56 formalin-fixed, paraffin-embedded tissue blocks from 41 patients with superficial bladder cancer and 15 with invasive bladder cancer. The 46 male and 10 female patients had a mean age of 67 years, with range of 40 to 82 years. The mean follow-up time was 33.4 (range 5-120) months. Tumor grade 1:2:3 ratios were 5:32:19. In superficial bladder tumor, abnormal expression of E-cadherin and  $\alpha$ -catenin was demonstrated in 37

and 71% of the tumors, respectively. In advanced bladder tumor, abnormal expression of E-cadherin and  $\alpha$ -catenin was demonstrated in 80 and 100% of the tumors, respectively. Differences in expression of E-cadherin and  $\alpha$ -catenin could be discerned between superficial and advanced bladder tumors ( $p=0.004$ ,  $p=0.024$ , respectively). However, the association between E-cadherin and  $\alpha$ -catenin expression and tumor grade was not statistically significant ( $p>0.05$ ). In addition, the expression of E-cadherin and  $\alpha$ -catenin did not correlate with tumor number and size ( $p>0.05$ ). We have demonstrated that abnormal expression of E-cadherin and/or  $\alpha$ -catenin occurs in more than 85% of bladder carcinomas and correlates significantly only with advanced stage. Nevertheless, these observations need to be confirmed in larger prospective clinical studies. (Pathology Oncology Research Vol 12, No 1, 13-19)

*Key words:* E-cadherin,  $\alpha$ -catenin, transitional cell carcinoma, urinary bladder

### Introduction

Transitional cell carcinoma (TCC) of the bladder is the fifth most common solid malignancy in the United States, and is diagnosed in approximately 54,000 patients and results in 12,000 deaths annually.<sup>10</sup> Bladder cancer is characterized by a diverse biological behavior, in which acquisition of the metastatic capacity is clinically most relevant. Although tumor grade and pathological stage have been the cornerstones of predicting outcome for bladder cancer, a variety of tumor markers and prognostic factors have

recently been used to study the progression of transitional cell malignancies.<sup>13</sup> Recently, the role of cell adhesion molecule expression in tumor invasion and metastasis has received significant consideration as a potential indicator of the metastatic phenotype in a variety of epithelial and nonepithelial malignancies.<sup>8,24,25</sup>

The cadherins are a family of transmembrane glycoproteins that mediate homophilic calcium-dependent intercellular adhesion, and E-cadherin is the major cadherin molecule expressed by epithelial cells.<sup>30</sup> Cadherins form complexes with cytoplasmic proteins, called catenins, which comprise three molecules:  $\alpha$ -,  $\beta$ -, and  $\gamma$ -catenins. The linkage of E-cadherin and catenins is necessary for the formation of strong intercellular adhesion. Recent studies have revealed that  $\beta$ -catenin and  $\gamma$ -catenin bind directly to the cytoplasmic domain of E-cadherin, and that  $\alpha$ -catenin links the bound  $\beta$ -catenin to the actin microfilament net-

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work of the cellular cytoskeleton.<sup>23,27</sup> Several studies have demonstrated that decreased expression of E-cadherin, as determined by immunohistochemistry, is associated with high grade and advanced stage in transitional cell carcinoma of the bladder.<sup>3,4,26,28,29</sup> Furthermore, altered E-cadherin expression has been shown to be associated with decreased recurrence-free and overall survival, as well as increased disease progression.<sup>12,21</sup> Yet, only a few studies have been carried out to examine the relationship between well-known bladder cancer prognostic factors and E-cadherin and catenin by using formalin-fixed and paraffin-embedded bladder carcinoma specimens.<sup>15,16,26,28</sup>

The objective of this study was to investigate E-cadherin and  $\alpha$ -catenin expression in superficial, noninvasive papillary TCC and invasive TCC, and correlate these results with pathological and clinical parameters.

### Materials and Methods

#### *Patients and tumor specimens*

Formalin-fixed, paraffin-embedded tissue blocks were obtained from 56 patients who underwent surgery for superficial and invasive bladder cancer at the Akdeniz University Hospital, Antalya, Turkey. Of these 56 cases, 41 cases were superficial TCC, and 15 were invasive TCC. Tumor grade 1:2:3 ratios were 5:32:19. The 46 male and 10 female patients had a mean age of 67 years, with range of 40 to 82 years. The mean follow-up time was 33.4 (range 5-120) months. All patients underwent transurethral resection (TUR) of the primary tumor. Blocks were selected by viewing original pathologic slides. The patient characteristics including sex, age at the time of surgery, histologic grade and stage, presence of carcinoma *in situ* (CIS), and cystoscopy features (tumor size, location, multiplicity) were obtained by examining medical records. No patient had received anticancer therapy prior to the operation. The staging was performed according to the 1997 TNM classification, while the grading was based on the World Health Organization (WHO) classification.<sup>7,18</sup> Recurrence was defined as any evidence of tumor in a retained bladder at least 3 months after treatment. Disease progression was defined as the development of invasive carcinoma (stage pT1 or higher) when initial diagnosis was pTis, pTa, or the development of muscle-invasive carcinoma (stage pT2 or higher) when initial diagnosis was pT1. Cystoscopy was performed every 3 months for 2 years after TUR, then every 6 months from the 3<sup>rd</sup> to 4<sup>th</sup> years and annually after 4 years.

#### *Immunohistochemistry*

Rabbit polyclonal IgG antibodies to E-Cadherin (H-108) and  $\alpha$ -catenin (H-297), all packaged at 200  $\mu$ g/ml, were purchased from Santa Cruz Biotechnology, Inc (USA). Appropriate antibody dilutions were determined by serial

titration in the presence of positive and negative controls. Anti-E-cadherin and anti- $\alpha$ -catenin final dilutions were 1:200. To enhance the immunoreactivity in formalin-fixed, paraffin-embedded tissues, sections were treated with an antigen retrieval solution in a microwave oven. Briefly, the slides were submerged in 0.01 M citrate buffer at pH 6.0 and heated to 90 °C for 30 minutes, pausing to ensure there was no fluid loss due to evaporation. Slides were then rinsed in phosphate-buffered saline (3x5 minutes) after each stage.

Fifty  $\mu$ l anti-E-cadherin or anti- $\alpha$ -catenin antibody was then added to the section and incubated for 2 hours at room temperature. After washes with PBS (3x5 min), the sections were incubated with Labvision polyvalent detection kit (TP-125-HL) and DAB (K-3466; Dako, Denmark). Slides were then washed and mounted for microscopic examination. Normal epithelial bladder tissue present in the tumor slides was used as internal positive controls.

#### *Evaluation of E-cadherin and $\alpha$ -catenin expression*

Assessment of the staining results was evaluated by one observer without knowledge of the clinical data such as tumor stage, grade, and survival. The expression of E-cadherin and  $\alpha$ -catenin in cancer cells was compared with that of normal epithelial cells in the same sample. In accordance with previously published criteria,<sup>9,20</sup> cancer cells that were stained at least as strongly as normal epithelial cells were defined as positive. E-cadherin and  $\alpha$ -catenin expression in the tumors was graded according to the proportion of positive cells. When >90% of the cancer cells were positively stained, the tumors were considered to be uniformly positive; when 10-90% of the cells were stained, the tumors were considered heterogeneous; when 0-10% of the cells were stained, the tumors were considered to be focally positive. Uniformly positive staining was regarded as normal, whereas heterogeneous, focally positive, uniformly negative and cytoplasmic staining was scored as aberrant.

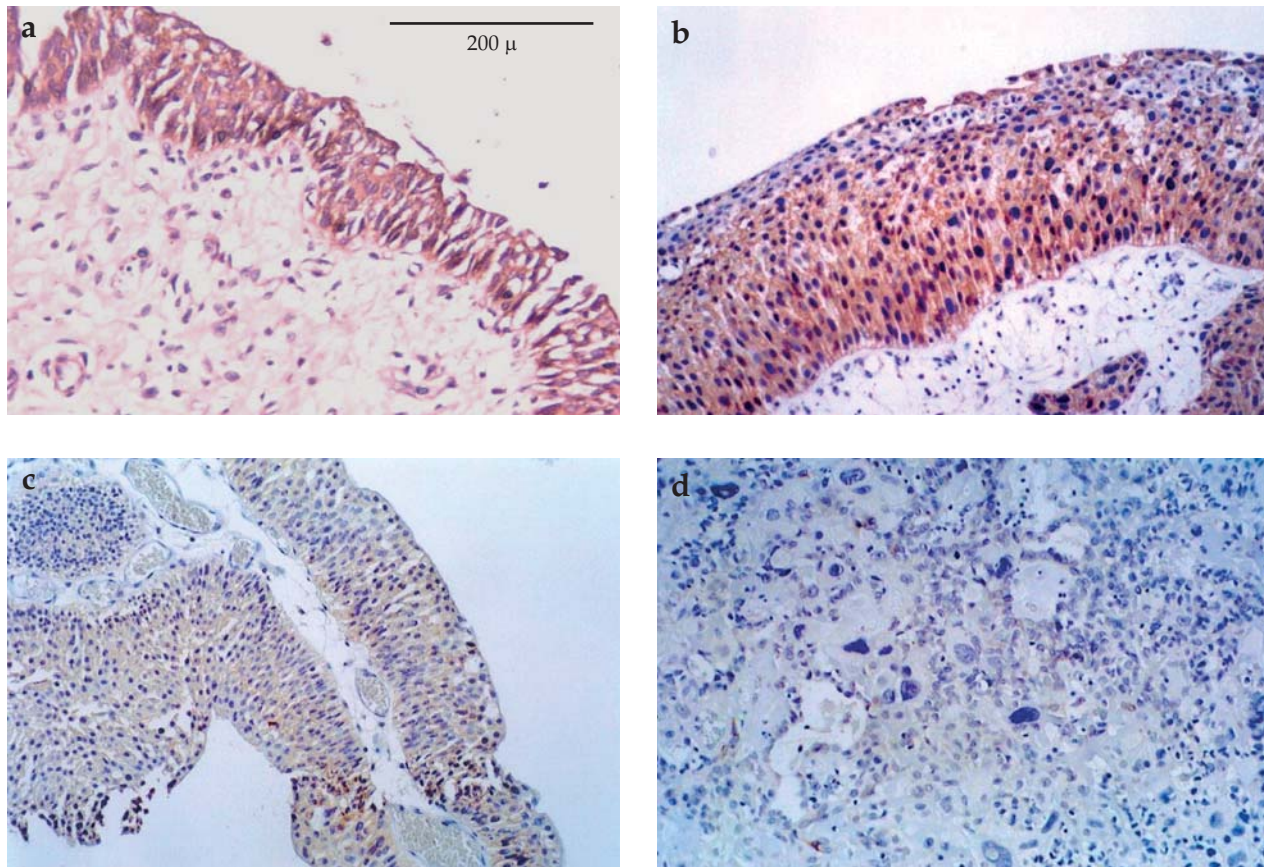
#### *Statistical analysis*

SPSS 10.0 software was used for statistical analysis. Disease-free survival was compared using Kaplan-Meier Survival analysis and log rank test. Chi-square test was used for comparing other parameters. A p value of less than 0.05 was accepted as statistically significant.

### Results

#### *Expression of E-cadherin and $\alpha$ -catenin in the normal bladder epithelium*

All of the noncancerous bladder epithelia showed equally strong membranous expression of E-cadherin and  $\alpha$ -catenin at the cell-cell boundaries, reflecting the normal localization of intercellular adhesion molecule; this served



**Figure 1.** E-cadherin immunoreactivity in normal bladder mucosa (a) and transitional cell carcinoma of the bladder (b-d); (b) positive, (c) heterogeneous, (d) negative. x200

as an internal positive control (Figures 1,2). Generally, normal bladder epithelium displayed strong staining for E-cadherin and  $\alpha$ -catenin. There was no detectable nuclear or stromal staining for either E-cadherin or  $\alpha$ -catenin in any section of normal bladder epithelium.

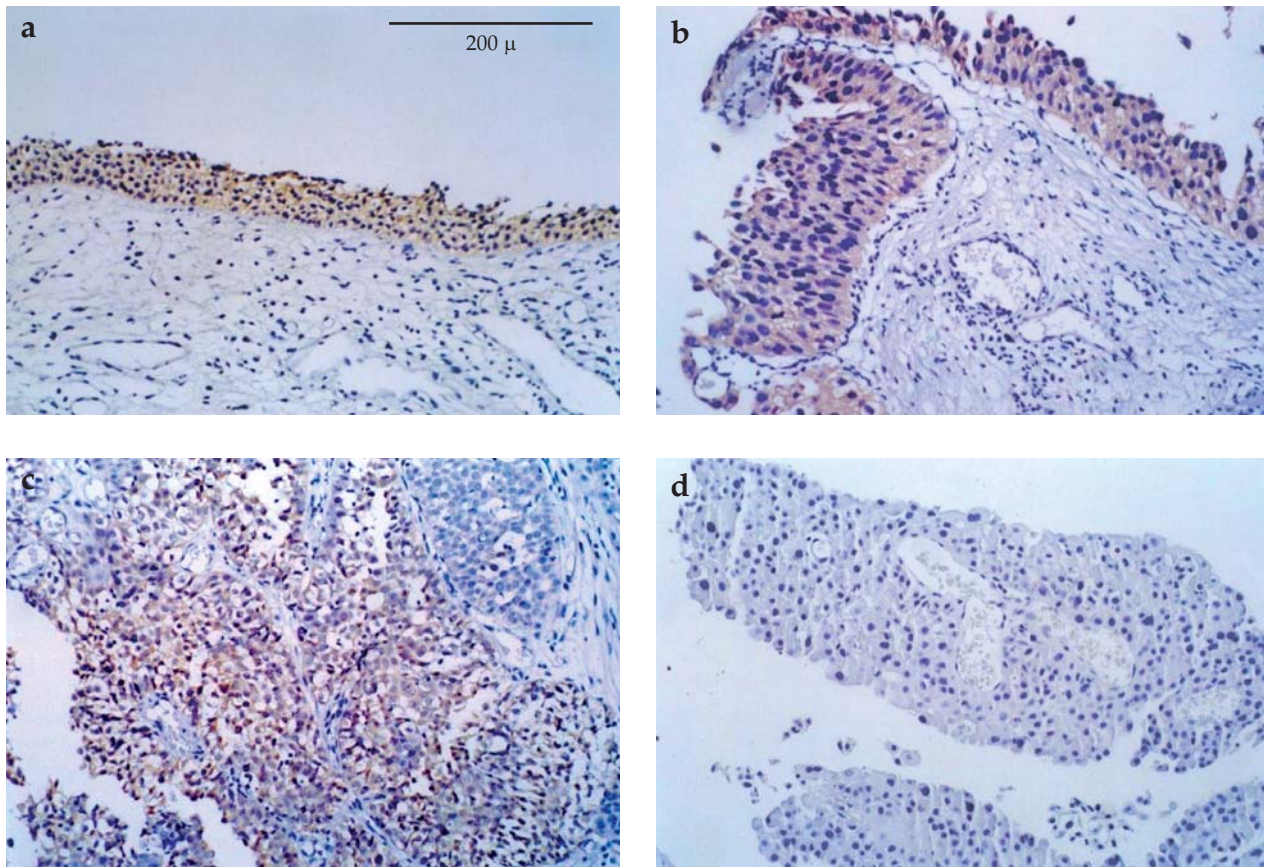
#### *Expression of the E-cadherin and $\alpha$ -catenin in bladder tumors*

In cancer cells, positive immunoreactivity for E-cadherin and  $\alpha$ -catenin was predominantly associated with cell-cell boundaries as in normal bladder epithelium (Figures 1,2). Heterogeneous immunoreactivity was expressed as a mixture of positive and negative cells, while negative immunoreactivity was expressed as trace amounts of E-cadherin and  $\alpha$ -catenin (Figures 1,2). In superficial bladder tumor, abnormal expression of E-cadherin and  $\alpha$ -catenin was demonstrated in 37 and 71% of the tumors, respectively. In advanced bladder tumor, abnormal expression of E-cadherin and  $\alpha$ -catenin was demonstrated in 80 and 100% of the tumors, respectively. Differences in expression of E-cadherin and  $\alpha$ -catenin could be discerned between superficial and advanced bladder tumors

( $p=0.004$ ,  $p=0.024$ , respectively). However, the association between E-cadherin and  $\alpha$ -catenin expression and tumor grade was not statistically significant ( $p>0.05$ ) (Table 1). In addition, no association was noted between E-cadherin and  $\alpha$ -catenin expression ( $p=0.364$ ) (Table 2).

#### *Correlation between E-cadherin and $\alpha$ -catenin expression and tumor number or size*

In solitary tumors, abnormal expression of E-cadherin and  $\alpha$ -catenin was demonstrated in 47 and 76% of the cases, respectively. In multiple tumors, abnormal expression of E-cadherin and  $\alpha$ -catenin was demonstrated in 49 and 79% of the cases, respectively. The expression of E-cadherin and  $\alpha$ -catenin did not correlate with the number of tumors ( $p>0.05$ ) (Table 1). In tumors  $< 3$  cm in maximum diameter, abnormal expression of E-cadherin and  $\alpha$ -catenin was demonstrated in 50 and 64% of the cases, respectively. In tumors  $\geq 3$  cm in maximum diameter, abnormal expression of E-cadherin and  $\alpha$ -catenin was demonstrated in 46 and 93% of the cases, respectively. The expression of E-cadherin and  $\alpha$ -catenin showed no correlation with tumor size ( $p>0.05$ ) (Table 1).



**Figure 2.**  $\alpha$ -catenin immunoreactivity in normal bladder mucosa (a) and transitional cell carcinoma of the bladder (b-d); (b) positive, (c) heterogeneous, (d) negative. x200

#### *Disease-free survival according to E-cadherin and $\alpha$ -catenin expression*

Recurrence was identified in 14 (25%) of patients, and immunohistochemical examination of their specimens revealed abnormal staining patterns of E-cadherin and/or  $\alpha$ -catenin molecule in all cases. The mean recurrence time of the tumors was  $19.3 \pm 11.84$  months, ranging from 5 to 42 months. No progression was identified in these patients. Abnormal E-cadherin and  $\alpha$ -catenin expressions were not associated with increased tumor-free survival ( $p > 0.05$ ) (Figure 3).

#### **Discussion**

Transitional cell carcinoma of the urinary bladder comprises a spectrum of diseases with diverse natural histories. Seventy to 80% of these tumors present as superficial lesions that recur in 30-90% of the patients.<sup>27</sup> Fifteen to 20% of these recurrences become invasive/metastatic.<sup>11</sup> Patients with invasive carcinomas are usually treated by radical cystectomy. However, metastatic disease appears after surgery in approximately 50% of the cases.<sup>31</sup> There-

fore, it is important to identify patients who might benefit from surgery with or without adjuvant therapy, including chemotherapy and radiotherapy. Several biological and molecular parameters have been considered as potential prognostic markers for bladder cancer but, up to now, tumor grade and stage have been the most important prognostic variables.<sup>19</sup> There is, however, significant intra- and interobserver variation in the reporting of tumor grade and stage. More reliable and objective indicators of prognosis are required.<sup>1</sup>

E-cadherin is a cell adhesion molecule located on the long arm of chromosome 16 at position 22.1.<sup>14</sup> Based on the correlation between 16q deletion and abnormal staining patterns, mutations of the E-cadherin gene have been proposed.<sup>5</sup> E-cadherin would thus behave as a classical suppressor gene. The  $\alpha$ -,  $\beta$ - and  $\gamma$ -catenins link E-cadherin to the actin cytoskeleton and are important for maintaining its role in cell-cell adhesion. *In vitro*, cells lacking  $\alpha$ -catenin are unable to form stable adherent junctions despite normal E-cadherin- $\beta$ -catenin expression and, when  $\alpha$ -catenin expression is restored by cDNA transfection, the transfected cells form normal epithelial structures.<sup>6</sup> Loss of E-cadherin expression associated with aggressive behavior

**Table 1. E-cadherin and  $\alpha$ -catenin staining patterns according to clinicopathological features in archival paraffin-embedded tissues from 56 patients with transitional cell carcinoma of the bladder**

	N (%)	E-cadherin		$\alpha$ -catenin	
		Normal (%)	Abnormal (%)	Normal (%)	Abnormal (%)
<i>Grade</i>					
1	5 (9)	3 (60)	2 (40)	0 (0)	5 (100)
2	32 (57)	17 (53)	15 (47)	9 (28)	23 (72)
3	19 (34)	9 (47)	10 (53)	3 (16)	16 (84)
			p>0.05		p>0.05
<i>Stage</i>					
Superficial	41 (73)	26 (63)	15 (37)	12 (29)	29 (71)
Invasive	15 (27)	3 (20)	12 (80)	0 (0)	15 (100)
			p=0.004		p=0.024
<i>Tumor number</i>					
Solitary	17 (30)	9 (53)	8 (47)	4 (24)	13 (76)
Multiple	39 (70)	20 (51)	19 (49)	8 (21)	31 (79)
			p>0.05		p>0.05
<i>Tumor size</i>					
< 3 cm	28 (50)	14 (50)	14 (50)	10 (36)	18 (64)
$\geq$ 3 cm	28 (50)	15 (54)	13 (46)	2 (7)	26 (93)
			p>0.05		p>0.05

has been described in a variety of human neoplasms. Similarly, it has been reported that loss of the invasion suppressor molecule E-cadherin is associated with deeply invasive, high-grade and advanced stage bladder cancers.<sup>3,24,28</sup> We found that 37% of superficial tumors and 80% of advanced tumors showed abnormal E-cadherin immunoreactivity. We have confirmed results of previous studies by demonstrating that E-cadherin expression is associated with tumor invasiveness, as represented by the pathological stage.<sup>3,24,28</sup> However, in our study, E-cadherin expression was not associated with tumor grade. While most reports have shown E-cadherin staining to be associated with tumor grade, others have not.<sup>3,12,15,24</sup> To our knowledge, only a few studies, yielding controversial results, have addressed  $\alpha$ -catenin expression in bladder cancers.<sup>15,16,26,28</sup>

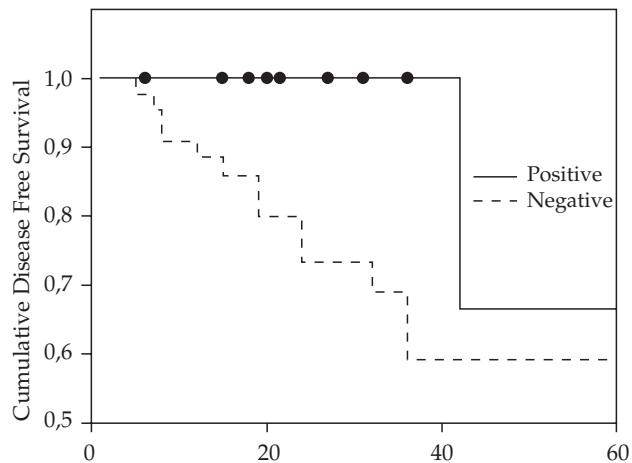
Our data suggest that immunostaining for  $\alpha$ -catenin is an accurate indicator of dysfunction. This is supported by our finding that the loss of  $\alpha$ -catenin staining in the presence of normal E-cadherin expression is frequent. We found no correlation between E-cadherin and  $\alpha$ -catenin expression. This observation suggests that expression of  $\alpha$ -catenin more directly reflects loss of cell-cell adhesion than does E-cadherin alone. The mechanism underlying this lack of  $\alpha$ -catenin staining despite normal E-cadherin expression has not been resolved. The loss of  $\alpha$ -catenin could be causally related to impaired catenin function, i.e., reintroducing neural  $\alpha$ -catenin in the PC9 carcinoma cells lacking  $\alpha$ -catenin restores calcium-dependent adhesiveness and results in reversion of morphology.<sup>6</sup> Hence, it is tempting to speculate that  $\alpha$ -catenin can indeed function as an

invasion suppressor gene in a subset of bladder tumor patients. We found that 71% of superficial tumors and 100% of advanced tumors showed abnormal  $\alpha$ -catenin immunoreactivity, which was associated with advanced tumor stage ( $p<0.05$ ). However, in our study  $\alpha$ -catenin expression was not associated with tumor grade. Recent studies have shown that either abnormal E-cadherin or catenin expression was strictly related to clinicopathological data in bladder tumors.<sup>16,26,28</sup> Mialhe *et al.* reported that the E-cadherin-catenin complex ( $\alpha$ -,  $\beta$ -,  $\gamma$ -catenin) was directly related to increasing tumor grade and deep invasion.<sup>16</sup> A significant correlation between normal expression of E-cadherin and normal expression of each catenin types ( $\alpha$ -,  $\beta$ -,  $\gamma$ -catenin and p120<sup>cas</sup>) in 48% of the cases studied was shown.<sup>26</sup> On the contrary, Syrigos *et al.* reported that only 4.4% patients with bladder tumor had normal expression for all four components of the E-cadherin-catenin complex ( $\alpha$ -,  $\beta$ -,  $\gamma$ -catenin).<sup>28</sup> We found that 8/56 (14%) of our patients had normal expression of E-cadherin and  $\alpha$ -catenin. Bringuier *et al.* reported that comparison of Northern blotting and immunohistochemistry in a series of

**Table 2. Correlation between E-cadherin and  $\alpha$ -catenin expression\***

E-cadherin	$\alpha$ -catenin	
	Normal	Abnormal
Normal	8	21
Abnormal	4	23

\*p = 0.364



**Figure 3.** Kaplan-Meier survival plot of disease-free survival according to  $\alpha$ -catenin positivity. Dots and triangles represent censored cases ( $p=0.146$ , log rank test).

49 frozen bladder tumors revealed clear mRNA down-regulation in 16 of 23 tumors with abnormal staining. In the 7 cases without mRNA down-regulation, no structural anomalies of E-cadherin could be detected by Southern blotting, Western blotting or PCR-SSCP. Western blotting confirmed that, in 6 of these tumors, E-cadherin was down-regulated at the protein level. This down-regulation was accompanied by down-regulation of  $\alpha$ -catenin and, to a lesser extent, of  $\beta$ - or  $\gamma$ -catenin. However, Northern-blot analysis indicated that expression of the 3 catenins was maintained at the mRNA level. Their data showed that, in bladder tumors, mRNA down-regulation accounts for about two thirds (16/23) of tumors with abnormal staining, and that post-transcriptional down-regulation of E-cadherin occurs in 6/23 of these tumors. The major mechanism associated with abnormal E-cadherin staining in bladder tumor is mRNA down-regulation. Loss of expression of one of the catenins at the mRNA level is not frequent. Besides E-cadherin mRNA down-regulation, functional regulation of the cadherin-catenin complex with down-regulation of E-cadherin at the protein level can also occur.<sup>2</sup>

Stage, grade, age of the patients, tumor size and pathologic architecture, dysplasia, multiplicity, time interval to recurrence, concomitant carcinoma *in situ* (Cis) and hydronephrosis in bladder cancer can all be used as classic clinical prognostic parameters. Recently, a multivariate analysis of the prognostic factors of primary superficial bladder cancer confirmed that multiple tumors, tumors greater than 3 cm, grade 3 tumors and Cis increased the risk of recurrence and progression.<sup>17</sup> We investigated the relationship between E-cadherin and  $\alpha$ -catenin expression and clinical features, such as tumor multiplicity and size. We found that the expression of E-cadherin and  $\alpha$ -catenin

did not correlate with tumor multiplicity and size ( $p>0.05$ ). In our study, recurrence was identified in 25% of patients and immunohistochemical examination of their specimens revealed abnormal staining patterns of E-cadherin and/or  $\alpha$ -catenin molecule in all cases, and no progression was identified in these patients. We found that abnormal E-cadherin and  $\alpha$ -catenin expression was not associated with increased probability of tumor-free survival ( $p>0.05$ ).

In conclusion, we have demonstrated that abnormal expression of E-cadherin and/or  $\alpha$ -catenin occurs in more than 85% of bladder carcinomas, and correlates significantly only with advanced stage. Nevertheless, these observations need to be confirmed in larger prospective clinical studies.

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