Expression of E-Cadherin and α-, β-, γ-Catenins in Patients With Bladder Cancer

Identification of γ-Catenin as a New Prognostic Marker of Neoplastic Progression in T1 Superficial Urothelial Tumors

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Abstract

*Loss of intercellular adhesion facilitates tumor invasion. To clarify the relation between altered expression of cell adhesion molecules and progression of T1 superficial bladder tumors, 101 cases (71 T1 tumors, 30 T2/T3 tumors) were examined immunohistochemically for E-cadherin and α-, β-, and γ-catenins. A highly significant correlation was observed between the decreased expression of all molecules and increased TNM stage (*P < .001*). Univariate analysis, performed in cases of T1 tumors, revealed association of abnormal E-cadherin with β-catenin diminution. Survival curves were established with the Kaplan-Meier method and analyzed according to clinical and histopathologic parameters using the log-rank test. Cox multivariate analysis revealed only γ-catenin as an independent predictor of progression-free survival in patients with stage T1 bladder urothelial tumors. The characterization of T1 tumors that will progress could lead to the identification of patients who might benefit from surgery to avoid vesical muscle invasion and, consequently, metastasis.*

Urothelial carcinoma is the most common solid malignancy of the bladder. It is distinguished as muscle-invasive carcinoma or superficial bladder tumor. Muscle-invasive carcinomas generally are associated with poor prognosis, whereas the clinical outcome of superficial bladder tumors is relatively unpredictable. The recurrence rate of T1 superficial bladder tumors is high (80%), and 40% of them will progress to a poorer prognosis muscle-invasive disease. Stage and grade, based on histopathologic criteria, are classic prognostic variables that permit evaluating recurrence or progression of the disease, which is useful for clinical purposes.[1] The identification of new prognostic markers allowing improvement of the biologic assessment of the T1 superficial tumors could be of great clinical value for disease management.

Tumor progression is accompanied by altered expression of cell adhesion molecules such as cadherins. E-cadherin, one subtype of transmembrane glycoprotein that mediates calcium-dependent adhesion of cells, is expressed specifically in epithelia and is involved in maintenance of their phenotype. Its cytoplasmic domain associates with cytoplasmic proteins termed catenins. β-Catenin or γ-catenin binds directly, whereas α-catenin links the bound β-catenin or γ-catenin to the actin microfilament network of the cellular cytoskeleton. This binding is essential for stable cell-to-cell adhesion.[2] Reduction in expression of these molecules has been reported in human cancers.[3-5]

In the bladder, studies have shown that decreased E-cadherin expression was linked to a loss of differentiation[6,7] and tumor aggressiveness,[8,9] and loss of E-cadherin expression was correlated with high grade and advanced stage.[10] Few studies have compared the predictive value of this adhesion molecule with that of clinical and pathologic parameters. E-cadherin status seems to be an independent predictor of disease

progression in patients treated with cystectomy for urothelial carcinomas.[11] When urothelial carcinomas become invasive, E-cadherin expression decreases in direct proportion to the depth of invasion.[12] Results on the independent clinical value of E-cadherin immunostaining are controversial.[10,13,14] Reduced expression of E-cadherin also was observed in the poorly differentiated and invasive tumor–derived cells.[15] Few data are available on altered α-, β-, and γ-catenin expression and tumorigenesis in the bladder. Abnormal expression of these cadherin cytoplasmic partners correlated significantly with tumor grade, advanced stage, and poor survival.[16,17]

The present study was undertaken to examine a large series of 101 urothelial carcinomas from stage T1 to T2/T3 for the expression of the adhesion molecules E-cadherin and α-, β-, and γ-catenin. The objectives of this study were as follows: (1) establish the relationship between expression of these adhesion molecules and the clinical and pathologic parameters, (2) define within T1 tumors their value in distinguishing T1a (minimally invasive) from T1b (invasive) tumors, (3) evaluate their use as predictive factors in the progression of T1a and T1b tumors, and (4) assess their potential prognostic role within T1 superficial bladder tumors in predicting survival without progression. Early detection within T1 tumors of a subgroup that will progress might successfully identify potentially lethal lesions (T1b?) before they become muscle invasive, and this could help identify patients who might benefit from surgery to avoid vesical muscle invasion and, consequently, metastasis.

Materials and Methods

Cases and Clinical Follow-up

We studied cases diagnosed between March 1973 and October 1996. In the present study, 101 cases with transitional cell carcinoma (TCC) of the bladder were analyzed for expression of E-cadherin and its cytoplasmic partners α-, β-, and γ-catenin. They comprised 71 T1 tumor cases for which the muscularis propria was present and not invaded and 30 invasive tumors (T2, 13 cases; T3, 17 cases). Histologic features, grade, stage, and presence of carcinoma in situ (CIS) were determined by pathologic examination of the transurethral resection specimens and were confirmed by blinded rereview of the original cystoscopic biopsy slides.

The essential improvement of the histologic prognosis and the reproducibility of grading for papillary urothelial neoplasms of the bladder led to the introduction of a number of classification systems for the grading of these tumors. Indeed, the reproducibility of grading has been discussed. The International Society of Urological Pathology and the World Health Organization (WHO) proposed new criteria for new classifications in 1998 and 1999, respectively. These classifications have not been approved by most pathologists who defended the refinement of the 1973 WHO classification[18] for grading. Thus, despite the absence of detailed defining criteria, the 1973 WHO classification has remained widely used.[19]

In this context, we assigned the bladder tumors a grade according to the 1973 WHO classification. The depth of invasion was recorded according to the 1997 TNM staging system guidelines.[20] Stage T1 (lamina propria invasion) has been divided into T1a (no muscularis mucosae invasion) and T1b (muscularis mucosae invasion),[21] which has a significantly higher risk of progression.[22] Clinicopathologic parameters of the tumor set and patient characteristics are described in **Table 1**. Before 1993, in our urology department, transurethral resection was the only standardized treatment for stage T1 urothelial tumors. Radical cystectomy was performed for large tumors (>5 cm) or grade 3 multifocal tumors or tumors associated with CIS. Patients with 2- to 5-cm, unifocal, grade 3 tumors and without associated CIS were treated by partial cystectomy and iridium 192 interstitial radiotherapy after the initial transurethral resection. After 1993, in addition to transurethral resection, intravesical bacille Calmette-Guérin instillations were systematically administered to patients with CIS associated with a grade 3 tumor. The median patient follow-up was 58.1 months (range, 5-188 months).

Table 1
Characteristics of 101 Patients With Bladder Urothelial Carcinomas

Characteristics	No. (%) of Patients
Age (y)	
<70	48 (47.5)
≥70	53 (52.5)
Sex	
Male	81 (80.2)
Female	20 (19.8)
Pathologic stage/histologic grade	
T1	71 (70.3)
G1	3 (4.2)
G2	30 (42.3)
G3	38 (53.5)
T2	13 (12.9)
G2	4 (30.8)
G3	9 (69.2)
T3	17 (16.8)
G2	1 (5.9)
G3	16 (94.1)
T1 subclassification	
T1a	46 (64.8)
T1b	25 (35.2)
T1/carcinoma in situ	
No	39 (54.9)
Yes	32 (45.1)
T2-T3/carcinoma in situ	
No	20 (66.7)
Yes	10 (33.3)
T1 tumor treatment	
Transurethral resection	50 (70.4)
Bacille Calmette-Guérin therapy	7 (9.9)
Partial cystectomy and interstitial radiotherapy	14 (19.7)

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Immunohistochemical Analysis

Tissue samples, obtained by endoscopic resection or partial cystectomy, were fixed in 4% formalin and paraffin embedded. Blocks were cut serially at 4 μm thick, deparaffinized in toluene, and rehydrated in graded ethanol. Antigen retrieval was achieved by microwave treatment in 0.5 mol/L of tris(hydroxymethyl)aminomethane-buffered saline (pH 6.0) at 750 W for 30 minutes. Sections were incubated with primary mouse antibodies at room temperature ▮**Table 2**▮ using an automated immunohistochemical processor (Techmate 500 Plus, DakoCytomation, Trappes, France) according to the manufacturer's instructions. Slides then were treated with biotinylated goat antimouse IgG (DakoCytomation) and avidin combined in vitro with horseradish peroxidase. Endogenous peroxidase activity was removed by dipping the sections in 5% hydrogen peroxide for 10 minutes at room temperature followed by incubation with streptavidin-horseradish peroxidase for 25 minutes. Finally, peroxidase activity was revealed by 3,3'-diaminobenzidine staining (0.9 mg/mL) for 15 minutes. Sections were counterstained with Harris hematoxylin, dehydrated through alcohol, and mounted using a standard procedure. Negative control samples were obtained by omitting the first antibody.

The status of the E-cadherin and catenins was assessed in a coded manner by a pathologist (A.C.) without knowledge of the clinical or pathologic features of the case. The proportion of stained cells and the cellular localization of immunostaining were used as criteria of evaluation. E-cadherin and α-, β-, and γ-catenin immunoreactivity was scored according to a classification derived from the work of Bringuier et al,[10] in which tumors were distinguished as *normal* if the staining was similar to that of normal urothelium. *Abnormal* tumors were defined as those with negative (ie, complete absence of immunoreactivity) or heterogeneous (ie, when the tumor is composed of positive and negative areas) staining and were considered 1 group in statistical analyses.

Statistical Analysis

Statistical analysis was carried out first on the entire group of cases (T1, T2, and T3 tumors). Differences in expression of E-cadherin and the 3 catenins according to patient and cancer characteristics were assessed by the χ^2 and Fisher exact tests. Second, survival without progression was studied on the T1 superficial bladder tumor subgroup. According to patient and cancer characteristics, curves were built and compared using the Kaplan-Meier procedure and the log-rank test. A Cox proportional hazards model then was used to select the independent prognostic factors from the variables found to be associated with survival without progression ($P < .20$) in the univariate analysis. The proportional hazards assumption was tested using time-dependent factors,[23] and a violation of this assumption was detected for the stage T1a/T1b variable. Therefore, the final Cox proportional hazards model was stratified on this factor. Statistical significance was set at the 5% level. Analyses were performed with SYSTAT 10 for windows (SPSS, Chicago, IL).

Results

Adhesion Molecule Study in T1 Superficial Bladder Tumors Compared With Muscle-Invasive Bladder Carcinomas

E-Cadherin Expression

Immunohistochemical analysis was performed on 101 TCCs of the bladder to identify the expression profile of E-cadherin. The main clinicopathologic features of our series of cases are enumerated in Table 1. ▮**Table 3**▮ summarizes the results of E-cadherin expression in T1 and T2/T3 bladder lesions. Of 71 T1 tumors, 69% had a similar staining pattern designated as normal staining (similar to normal urothelium, homogeneously observed at cell-cell borders), whereas 31% showed abnormal E-cadherin expression (heterogeneous or negative). Of these 31%, only 4% were completely negative and 27% were heterogeneous, with both positive and negative areas in the same tumor. Among the 30 T2/T3 tumors, only 33% showed normal expression of E-cadherin, and the prevailing abnormal pattern was heterogeneous (67%). No tumor was completely negative. In summary, the expression of E-cadherin was conserved on the whole in T1 superficial bladder tumors with the same immunostaining as normal urothelium and decreased in T2/T3 bladder carcinomas.

Table 2
Characteristics of Immunohistochemical Markers

Marker	Clone*	Source	Working Dilution	Incubation Time (min)
E-cadherin	HECD-1	Zymed Laboratories, Montrouge, France	1:600	30
α-Catenin	5	Transduction Laboratories, Lexington, KY	1:500	30
β-Catenin	14	Transduction Laboratories	1:500	30
γ-Catenin	15	Transduction Laboratories	1:500	30

* All were mouse monoclonal antibodies.

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Table 3
Relationship Between E-Cadherin/Catenin Expression and the Pathologic Stage (Superficial vs Invasive)*

	E-Cadherin and Catenin Immunostaining			
Tumor Stage	**Normal**	**Heterogeneous**	**Negative**	***P*†**
E-cadherin				
T1	69	27	4	.001
T2/T3	33	67	0	
α-Catenin				
T1	75	21	4	.001
T2/T3	33	63	4	
β-Catenin				
T1	75	25	0	.001
T2/T3	17	83	0	
γ-Catenin				
T1	88	8	4	.001
T2/T3	30	67	3	

* Data are given as percentages.
† Normal vs heterogeneous/negative.

α-, β-, and γ-Catenin Expression

Similar to E-cadherin, all catenins were immunodetected and localized to the membrane. Immunostaining for γ-catenin is shown in **Image 1**. At the normal urothelium, membranous γ-catenin expression was detected at the cell-cell borders (Image 1A). The luminal membrane and the parts of the cells in contact with the basement membrane did not react with the anti–γ-catenin antibody. Focal stronger reactivity was detected at the apical junctional complexes. Positive staining fits with immunostaining similar to that of normal urothelium (Image 1B). Heterogeneous staining (Image 1C) is related to the presence of positive and negative areas in the same tumor. Negative staining (Image 1D) squares with a complete absence of immunoreactivity. Table 3 summarizes the results of catenin expression in T1 and T2/T3 bladder lesions. High normal expression of all 3 cytoplasmic proteins was observed in T1 tumors (75%-88%), which decreased in T2/T3 tumors (17%-33%). In invasive tumors, abnormal expression of β-catenin was recorded most frequently (83% of cases), followed by γ-catenin (70% of cases) and α-catenin (67% of cases). As for E-cadherin, for each catenin, the prevailing abnormal staining was heterogeneous.

Statistical Analysis Comparing the Adhesion Molecules, Expression in T1 Tumors and in T2/T3 Tumors

This analysis revealed significantly reduced expression of adhesion molecules in muscle-invasive bladder carcinomas compared with T1 superficial bladder tumors ($P < .001$).

Adhesion Molecule Study Within T1 Superficial Bladder Tumors

Univariate Statistical Analysis

Univariate statistical analysis was performed in T1 tumors by comparing the expression of E-cadherin and α-, β-, and γ-catenins among themselves and the expression of each adhesion molecule with different clinical and anatomic-pathologic parameters. Comparison of E-cadherin expression results with classic clinical and histologic data revealed that abnormal E-cadherin expression is associated with decreased β-catenin expression ($P = .04$) but, it is important to note, not with grade ($P = .69$). Comparison of the expression of the α-, β-, and γ-catenins among themselves revealed that abnormal expression in a given catenin is associated significantly with the diminution of the other two (α vs γ, $P = .02$; α vs β, $P = .001$; β vs γ, $P = .02$). In addition, abnormal α-catenin expression is associated with high grade ($P = .02$). Similarly, abnormal β-catenin expression is associated with an abnormal pattern of E-cadherin expression ($P = .04$) but not with clinical or histologic parameters.

Among our histologic data, we introduced the subclassification T1a/T1b. Considering this subclassification, the results of the expression profile of E-cadherin and the α-, β-, and γ-catenins did not differ significantly according to the invasion of the muscularis mucosae **Table 4**. None of the markers examined distinguished T1a and T1b tumors.

Survival Analysis

Table 5 gives the results of progression-free survival univariate analysis. The progression-free survival curve is plotted according to expression of E-cadherin **Figure 1A** and γ-catenin **Figure 1B** within the 71 T1 superficial bladder tumors. Among all adhesion molecules studied, only γ-catenin was nearly significant ($P = .07$). Multivariate analysis revealed that only stage T1a/T1b and expression of γ-catenin remained significant. The relative risk of progression associated with a decreased expression of γ-catenin was 6.11 (95% confidence interval, 1.82-20.44; $P = .003$). Thus, γ-catenin seems to be a new independent prognostic marker of T1 superficial bladder tumor progression.

Discussion

Alteration of intercellular adhesion is a key event in the process of neoplastic cell growth that is associated with dedifferentiation and might facilitate tumor invasion and development of metastatic disease, finally affecting the survival of patients with cancer.

In keeping with the results of other studies,[10,11,13,17,24-26] we observed that reduced expression of E-cadherin was associated with depth of invasion. In our series, as observed in other studies,[10,11] most of the tumors with altered E-cadherin expression (98%) showed heterogeneous expression with both positive and negative areas in the same tumor. Heterogeneous E-cadherin staining likely could reflect tumor heterogeneity. However, heterogeneous E-cadherin expression also has been

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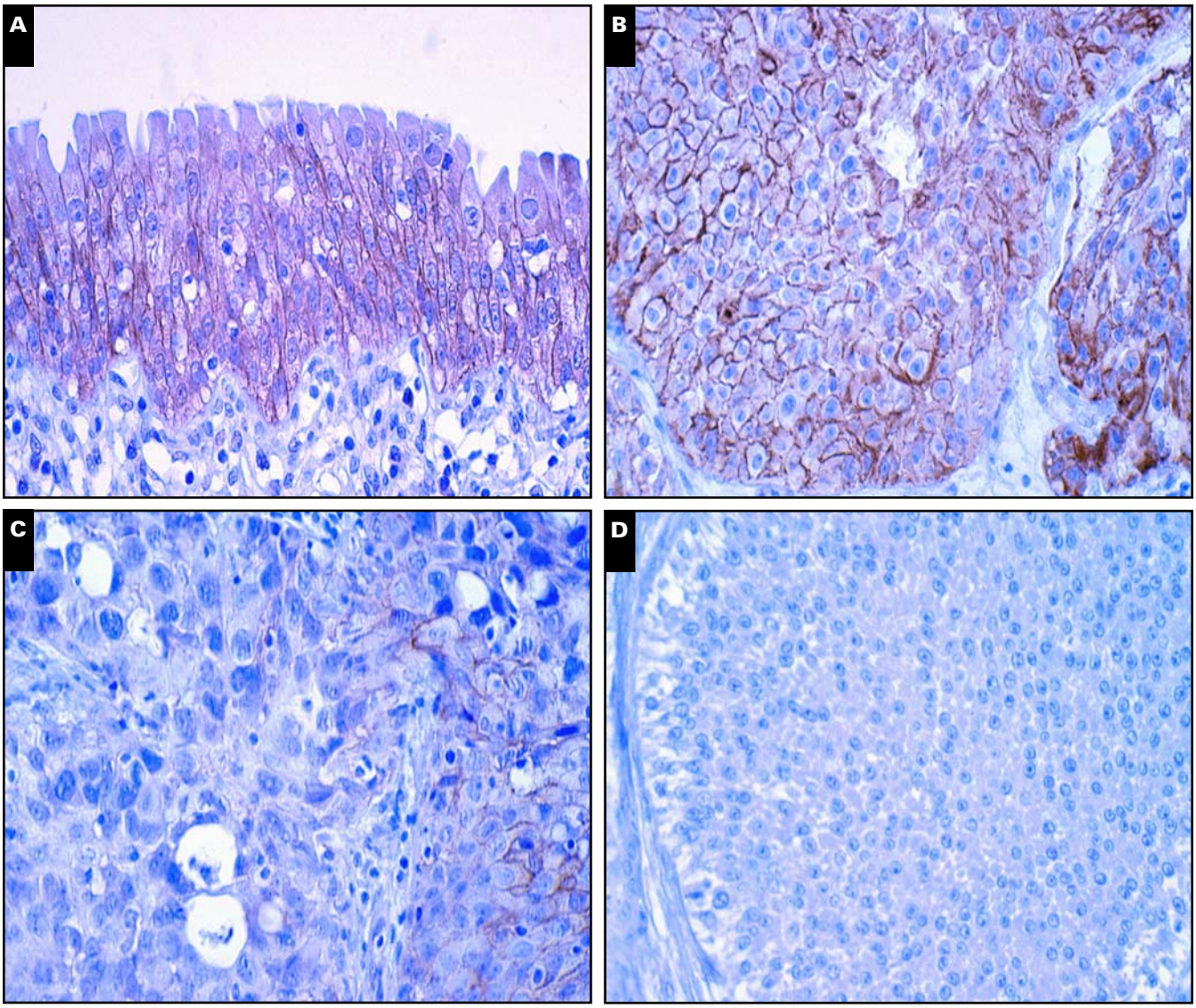

Image 1 Immunohistochemical staining of γ-catenin protein within T1 tumors. **A**, Positive membranous staining in normal urothelium (original magnification ×10). **B**, Transitional cell carcinoma of the bladder with a conserved staining at the cell-cell border. As in normal urothelium, a lack of immunoreactivity is observed along the cell surface in contact with the basement membrane (original magnification ×10). **C**, Heterogeneous staining in transitional cell carcinoma of the bladder. Some cells show clear staining at the cell-cell border, whereas other areas are negative (original magnification ×25). **D**, Negative staining in transitional cell carcinoma of the bladder. All cells are completely negative (original magnification ×10).

detected in a murine metastatic ovarian tumor cell line. It is thought to reflect unstable E-cadherin expression because this heterogeneity persists on subcloning.[27] In the same way, highly metastatic unstable E-cadherin expression that varied readily with cell culture conditions has been reported.[28] Thus, heterogeneous E-cadherin expression might be assigned not only to tumor heterogeneity but also to unstable expression in vivo. As in most reported studies,[11,13,17,25,29] we did not find any association between reduced E-cadherin expression and tumor grade, whereas others have shown E-cadherin staining to be associated with tumor grade.[10,24,30] In some studies, E-cadherin expression was associated with poor survival but was found in patients with all stages of tumors.[30] To our knowledge, we report for the first time that altered E-cadherin expression was not associated with the progression of T1 superficial bladder tumors.

Several molecular mechanisms may be involved in determining a reduction of E-cadherin expression in cancer cells such as alteration of transcription (methylation, loss of transactivators, chromatin rearrangement in the regulatory region, or down-regulation by specific repressors such as snail), post-translational modifications, or changes in the interaction of

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Table 4
Relationship Between E-Cadherin/Catenin Expression and the Subclassification T1a/T1b*

	T1a	T1b	*P*
E-cadherin			
Normal	67	72	.68
Abnormal	33	28	
α-Catenin			
Normal	80	64	.12
Abnormal	20	36	
β-Catenin			
Normal	74	76	.84
Abnormal	26	24	
γ-Catenin			
Normal	87	88	.90
Abnormal	13	12	

* Data are given as percentages.

Table 5
Univariate Progression-Free Survival Analysis in 71 Patients With T1 Bladder Urothelial Tumors According to the Kaplan-Meier Method and the Log-Rank Test

Variable	*P*
Age	.46
Sex	.14
E-cadherin	.86
α-Catenin	.32
β-Catenin	.71
γ-Catenin	.07
T1a/T1b	<.001
Grade	.11
Carcinoma in situ	.03

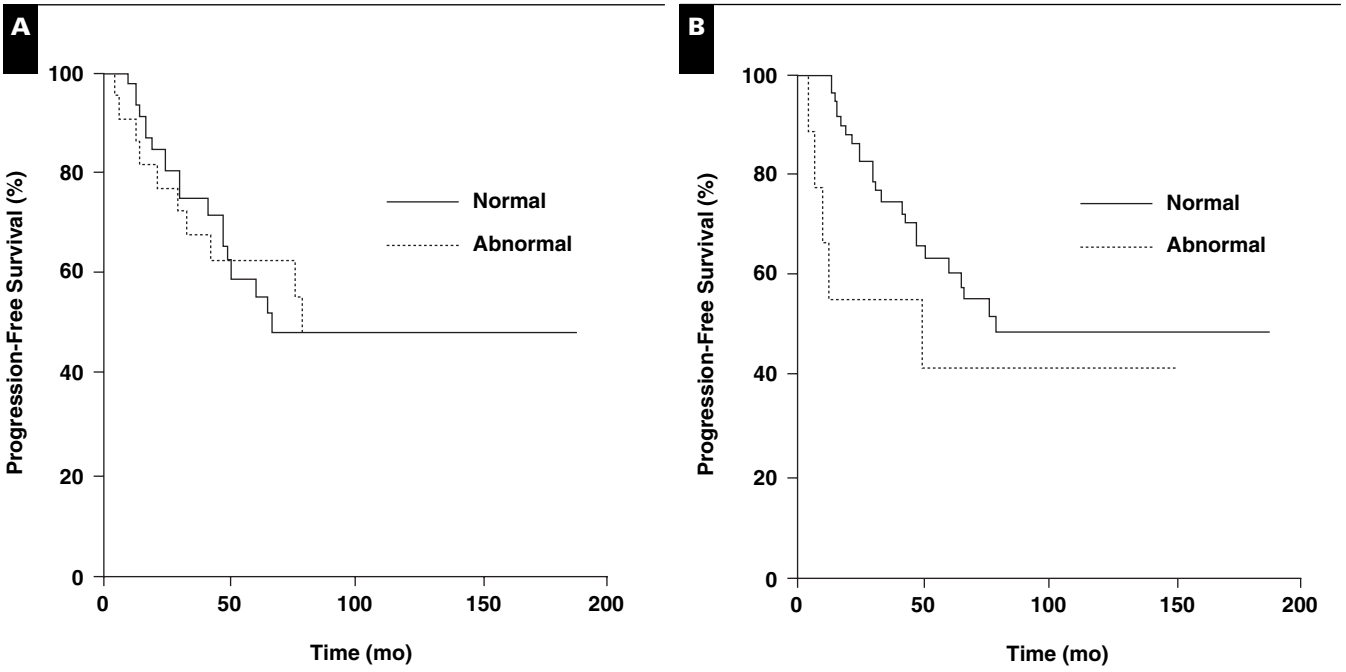

Figure 1 Kaplan-Meier progression-free survival curves according to E-cadherin expression (**A**) and γ-catenin expression (**B**) status in 71 cases of T1 superficial bladder tumors. The differences between groups were evaluated by the log-rank test. **A**, *P* = .86. **B**, *P* = .07.

E-cadherin with the cytoskeleton-anchoring catenin proteins.[31-35] Inactivating mutations of E-cadherin have been found commonly only in breast cancer of the lobular type and in the diffuse type gastric carcinomas.[36] A new mechanism involving endocytosis and subsequent degradation of E-cadherin has been shown to be mediated in part by an E3 ubiquitin ligase.[37]

It has been reported that association with catenins was a prerequisite for the cell adhesive activity of the classic cadherins. In fact, the presence of E-cadherin does not attest its functionality. We have observed concomitant expression of α-, β-, and γ-catenins and E-cadherin in T1 tumors, suggesting functional intercellular adhesion complexes. In the T2/T3 tumor group, decreased expression of the 3 catenins was associated with that of E-cadherin.

As for E-cadherin, the reduced β-catenin expression was not correlated with progression. Garcia del Muro et al[38] demonstrated that the simultaneous loss of E-cadherin and β-catenin expression predicted poor survival in patients with bladder cancer of all stages. Whatever the tumor stage in our series, no tumor was β-catenin–deficient, which suggests a crucial biologic role of this protein in bladder tissue. Indeed, β-catenin is a multifunctional protein—in addition to its function in adhesion complexes, β-catenin has a role

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in signal transduction pathways,[39] and in some tumors, these pathways are considered to be related to proliferation and invasion.[40] In invasive tumors, the decreased β-catenin expression was prevalent compared with α- and γ-catenins. This decreased membranous β-catenin expression could be ascribed to the turnover increase of the protein via the proteasome or its nuclear localization. The persistent β-catenin expression, even in the absence of E-cadherin expression in invasive tumors, could allow the formation of an intercellular adhesion complex involving another member of the cadherin family or could be due to the presence of a cytoplasmic pool of the protein. Besides, in grade 3 bladder cancers, Shiina et al[41] demonstrated that a β-catenin mutation led to its cytoplasmic accumulation and its localization to the nucleus with, consequently, increased expression of c-myc and cyclin D1.

Decreased α-catenin expression was associated only with grade 3 in our study in patients with T1 superficial bladder tumors and was not correlated with progression. Mialhe et al[17] previously demonstrated that abnormal α-catenin expression was associated with poor survival, but it was in patients with invasive lesions.

Few studies have explored the prognostic value of γ-catenin expression in bladder cancer.[16,26] The cellular localization of γ-catenin was studied by immunohistochemical analysis in 68 TCCs. There was a significant correlation between the loss of normal membranous expression of this molecule and increased grade. Furthermore, a highly significant correlation was observed between the loss of expression of γ-catenin with increased TNM stage. Moreover, the results suggested that abnormal γ-catenin expression was correlated with poor survival,[26] but in another study, it had a somewhat lower predictive value than E-cadherin and α- and β-catenins.[16] In our study, we showed that γ-catenin expression decreased according to the TNM stage. Moreover, in our series of T1 tumor samples, the evaluation of the prognostic value of γ-catenin expression and multivariate statistical analysis allowed us to identify this molecule as a new prognostic factor for tumor progression.

None of the markers studied allowed us to distinguish patients with T1a or T1b urothelial bladder tumors. In our study, the depth of invasion in T1 tumors seemed not to be a criterion to predict the progression of these superficial bladder tumors. A larger cohort of patients with T1a or T1b tumors should be studied to clarify this point. Nevertheless, to our knowledge, this is the first report linking γ-catenin expression to progression-free survival, thus identifying this adhesion molecule as a predictive factor for T1 tumor progression. This confirms the relevance of cell adhesion molecule expression to the clinical and biologic behavior of superficial bladder tumors. On these bases, further prospective studies may be undertaken to establish whether closer follow-up might be beneficial in patients with T1 superficial bladder tumors that express low levels of γ-catenin.

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