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Prognostic value of the expression of E-cadherin and β-catenin in bladder cancer

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Abstract

The purpose of this study was to assess the prognostic effect of the expression of E-cadherin, β-catenin and CD44 adhesion molecules in bladder carcinoma. 22 superficial and 18 invasive bladder tumour samples were studied by immunohistochemistry. The median follow-up was 24 months (range: 1–50 months). Loss of E-cadherin and β-catenin immunoreactivity was found in 14 (35%) and 17 (43%) tumours, respectively, and was significantly associated with invasiveness, high grade and p53 overexpression. There was no correlation between CD44 variant expression and clinicopathological findings. Loss of E-cadherin expression was an independent predictor of poor survival in a multivariate analysis, when assessed with age, grade, stage and p53 status (hazards ratio adjusted (HRa) = 4.45 [95% confidence interval (CI), 1.06–18.63]). This effect was particularly augmented in patients with invasive bladder cancer. When expression of E-cadherin and β-catenin were evaluated simultaneously, loss of immunoreactivity of both proteins was a strong predictor of poor survival (HRa = 13.06 [95% CI, 0.95–178.55]). The same pattern was found when progression-free survival in relation to these variables was assessed. In conclusion, assessment of E-cadherin and β-catenin immunoreactivity may be a useful prognostic marker in bladder cancer complementary to established prognostic factors.

Keywords: E-cadherin; β-catenin; CD44; Cell adhesion molecules; Bladder cancer; Prognosis

1. Introduction

Transitional cell carcinoma of the bladder is a heterogeneous disease. Most patients present with superficial tumours, and after endoscopic resection, the majority of them will develop further superficial recurrent tumours. However, up to one-third of patients will progress to invasive tumours. Amongst the patients with invasive tumours, only half will develop metastatic disease in subsequent years, after treatment with cystectomy or multimodal therapy. Stage and grade are classical prognostic variables that allowed a certain degree of stratification of tumour biological potential, useful for clinical purposes [1]. However, a considerable degree of heterogeneity still remains within the various prognostic subgroups, limiting the predictive value of these factors. The identification of new prognostic markers that allow improvements to be made in the biological assessment of bladder tumours could be of great clinical value [2].

Detachment of tumour cells from the primary lesion is considered a main step in the process of invasion of the surrounding tissues and metastases to distant organs [3]. Since E-cadherin and CD44 are cell surface adhesion molecules and β-catenin is an intracytoplasmic E-cadherin binding protein, loss of expression of these adhesion molecules may contribute to cancer progression. The present study was undertaken to investigate the alterations in the expression of E-cadherin, β-catenin, and CD44 in transitional cell carcinoma of the bladder, and their prognostic value.

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2. Patients and methods

40 patients with primary transitional-cell carcinoma of the bladder were included in this study. Patients were treated from March 1992 through to April 1995. The median age was 69 (range: 57–81 years), with 33 men and 7 women. Frozen tissue samples of the tumours, paraffin-embedded tissue blocks and clinical follow-up data were available in all cases. Tissue samples were obtained by radical cystectomy in patients with invasive tumours and cystoscopic resections in patients with superficial tumours. None of the patients received pelvic irradiation or systemic chemotherapy before surgery. The histological grading was performed according to UICC criteria. Patients were grouped as low grade (I and IIa) or high grade (IIb and III). The pathological staging was done according to the TNM classification. The tumours were grouped as superficial tumours (Ta and T1) and invasive tumours (T2, T3 and T4). Among the 40 tumours, 16 were classified as low grade (2 grade I and 14 grade IIa) and 24 as high grade (2 grade IIb and 22 grade III). 22 were classified as superficial tumours (7 Ta and 15 T1) and 18 as invasive tumours (8 T2, 6 T3 and 4 T4). The median follow-up was 24 months (range: 1–50 months).

2.1. Immunohistochemical evaluation

Four-micron sections from archival paraffin-embedded tissue were placed on poly-L-lysine coated slides (Sigma Chemical Co., St Louis, MO, USA). Cuts were dewaxed and rehydrated. Endogenous peroxidase inhibition was done by incubation in hydrogen peroxide (3% in methanol for 10 min) and rinsed in phosphate-buffered saline (PBS). For β-catenin, p53 and CD44 standard and v6 isoform detection, the microwave antigen retrieval method in sodium citrate (0.01 M, pH 6.0, for 10 min) was used. For E-cadherin detection, sections were pretreated with a solution of Pepsin Reagent (Biomeda, Foster City, CA, USA, M77) for 30 min at room temperature. For CD44v3–10 isoform detection, sections were pretreated with Triton X-100 (Sigma, 0.1% in blocking dilution for 90 min). For blocking, tissues were incubated with 3% horse serum in 2% PBS-bovine serum albumin (BSA) (20 min) (Sigma). Incubations were performed in a humidified chamber at 4°C. Washes were done in PBS. A biotinylated goat-antimouse secondary antibody was used at a dilution of 1:5000. Visualisation of the reaction was performed with an avidin–biotin complex immunoperoxidase system (Vector Laboratories, Burlingame, CA, USA) using diamino-benzidine (Sigma) as a chromogen and Mayer's haematoxylin as a counterstain. Finally, samples were dehydrated and mounted with xylene mountant DPX (BDH Lab, UK).

Antibodies used were the mouse monoclonal anti-E-cadherin (clone BTA-1) (RD Systems, Minneapolis, MN, USA) and anti-β-catenin (clone 14) (Transduction Laboratories, San Diego, CA, USA). CD44 expression was determined using the monoclonal antibody CD44s (SFF-2) that specifically recognises the standard form of CD44, monoclonal anti-CD44v6 (VFF-7) that reacts against an epitope of the human CD44 variant encoded by exon 6 and the polyclonal anti-CD44v3–10 that reacts against epitopes of the human variant transcribed by exons 3–10. p53 protein expression was determined using three different antibodies: anti-p53 DO-1, Pab1801 and anti-p53 MU-195. When p53 was positive for at least one antibody, it was defined as positive.

Evaluation of the staining was carried out by two investigators. CD44, E-cadherin and β-catenin membranous expression was evaluated in the major infiltrating zone of the tumour. Immunostaining for all the proteins showed a membrane pattern, except for the standard form of CD44, where staining was also found in the cytoplasm. The proportion of stained cells and the cellular localisation of immunostaining were used as criteria for the evaluation. Immunostaining was divided into three categories: positive homogeneous, when the pattern of immunoreactivity was membranous, similar to that of normal urothelial cells; positive focally, if tumours showed heterogeneous staining (20–80% positive tumour cells), and negative (less than 20% positive tumour cells or cytoplasmic or nuclear distribution). In the multivariate analysis, the categories 'positive homogeneous' and 'positive focally' were grouped as one.

2.2. Statistical methods

The probabilities of overall survival and progression-free survival were calculated using Kaplan–Meier estimates. Survival probability distributions were compared with the log-rank test (Mantel–Cox). Crude and adjusted hazard ratios were calculated using Cox's proportional hazards regression analysis. All Cox models appeared to appropriately fit the data. Statistical significance was established at the 0.05 α-value and accordingly 95% confidence intervals (CI) around hazard ratios are presented. All P values were derived from two-sided tests.

The association of E-cadherin and β-catenin immunoreactivity with grade, stage and p53 status was estimated by the chi-squared test.

3. Results

The expression of E-cadherin was positive homogeneous in 13 (33%) tumours, positive focally in 13 (33%) and negative in 14 (35%) (Fig. 1). Loss of membranous E-cadherin immunoreactivity was significantly associated with invasiveness ($P < 0.001$) and grade ($P < 0.001$) (Table 1). Positive homogeneous membrane

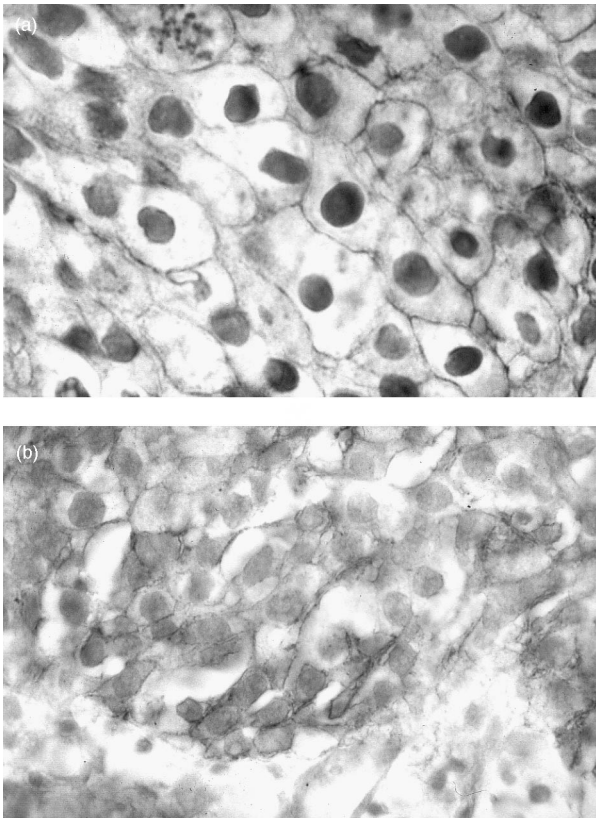

Fig. 1. Expression of E-cadherin and β-catenin in bladder cancer. (a) Grade II transitional-cell carcinoma showing positive membranous immunoreactivity for E-cadherin (×600). (b) Grade II transitional-cell carcinoma showing positive membranous immunoreactivity for β-catenin in the infiltrating front of the tumour (×400).

pattern for β-catenin was seen in 12 (30%) tumours. Eleven (28%) tumours were classified as positive focally and 17 (43%) were negative (Fig. 1). Loss of β-catenin immunoreactivity was statistically associated with grade ($P < 0.0001$) and invasiveness ($P = 0.001$) (Table 1). Normal urothelium was classified as positive homogeneous for E-cadherin and β-catenin in all studied patients. Nuclear accumulation of p53 was detected with at least one antibody in 15 (38%) tumours. The

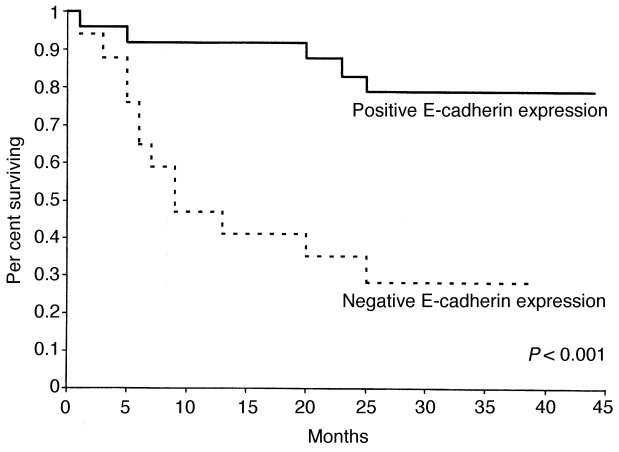

Fig. 2. Survival distribution in patients with bladder cancer according to E-cadherin expression status.

presence of nuclear p53 overexpression was significantly associated with E-cadherin and β-catenin immunoreactivity $P < 0.0001$ and $P = 0.002$, respectively (Table 1).

Standard CD44 was expressed on normal urothelium and by all but one transitional cell carcinoma. Variant forms of CD44 tested were positive in 33 (83%) and in 34 (85%) tumours for v6 and for v3–10, respectively. Variant forms were also seen in normal urothelium samples, especially when inflammatory changes were present. There was no association between CD44 variant expression and stage, grade, tumour progression and survival (data not shown).

Patients with loss of E-cadherin expression had a statistically significant decreased probability of overall survival ($P < 0.001$) (Fig. 2), as compared with patients with positive E-cadherin immunoreactivity. As shown in Table 2, in addition to E-cadherin status, tumour stage and grade were significantly associated with survival in the univariate analysis. In the multivariate analysis, E-cadherin expression was a significant independent predictor of survival, when simultaneously assessed with age, grade, stage and p53 status (Table 2). The subgroup of patients with invasive tumours was separately analysed in a multivariate analysis, and the prognostic

Table 1
Association of E-cadherin and β-catenin immunoreactivity with grade, stage and p53 status in 40 patients with bladder cancer

	E-cadherin			β-catenin		
	Positive *n* (%)	Negative *n* (%)	*P* value	Positive *n* (%)	Negative *n* (%)	*P* value
All patients	26 (65)	14 (35)		23 (58)	17 (43)	
Grade						
Low	16 (40)	0 (0)		15 (38)	1 (3)	
High	10 (25)	14 (35)	<0.0001	8 (20)	16 (40)	<0.0001
Stage						
Superficial	21 (53)	1 (3)		18 (45)	4 (10)	
Invasive	5 (13)	13 (33)	<0.0001	5 (13)	13 (33)	=0.001
p53						
Negative	22 (55)	3 (8)		19 (48)	6 (15)	
Positive	4 (10)	11 (28)	<0.0001	4 (10)	11 (28)	=0.002

Table 2
Risk of death of bladder cancer in relation to clinical, pathological and molecular characteristics

Characteristic	Patients (%)	No. of deaths	HRc (95% CI)	HRa (95% CI)
Age, years				
<70	20 (50)	5	1	1
$\geqslant 70$	20 (50)	12	2.59 (0.91–7.41)	2.13 (0.70–6.47)
Stage				
Superficial	22 (55)	4	1	1
Invasive	18 (45)	13	6.35 (2.03–19.92)	2.84 (0.79–10.23)
Grade				
Low (I–IIa)	16 (40)	1	1	1
High (IIb–III)	24 (60)	16	12.79 (1.66–98.23)	7.51 (0.70–80.32)
p53				
Negative	25 (63)	7	1	1
Positive	15 (38)	10	2.16 (0.78–6.01)	0.70 (0.24–2.07)
E-cadherin				
Positive	26 (65)	5	1	1
Negative	14 (35)	12	6.62 (2.22–19.7)	4.45 (1.06–18.63)
β-catenin				
Positive	23 (58)	5	1	1
Negative	17 (43)	12	4.62 (1.61–13.26)	1.75 (0.55–5.64)
E-cadherin and β-catenin				
Both positive	19 (48)	2	1	1
Either positive	11 (28)	6	6.44 (1.30–31.91)	3.80 (0.52–28.02)
Both negative	10 (25)	9	16.92 (3.50–81.75)	13.06 (0.95–178.55)

HRc, crude hazards ratio adjusted by age only; HRa, hazard ratio adjusted for age, stage, grade and p53 status; CI, confidence interval. Based on Cox proportional hazards model in univariate and multivariable analysis. CI that does not include the value of 1.00 indicates a significant association with overall survival at the $P=0.05$ level (two-sided).

impact of E-cadherin on survival approached statistical significance in spite of a low number of patients (hazards ratio (HR) = 5.83 [95% CI, 1.03–32.81]). Loss of expression of β-catenin was also associated with poor survival ($P=0.016$) (Fig. 3). However, in a multivariate analysis, β-catenin failed to show a correlation with survival, when factors such as stage, grade and p53 were entered into the model (Table 2).

When the combined expression of both variables E-cadherin and β-catenin was assessed, patients were classified into three categories: loss of expression of both variables, positivity of one of them with negativity of the other one and positivity of both variables. These categories were predictive of overall survival ($P<0.001$) (Fig. 4). In a multivariate analysis, negative expression of both E-cadherin and β-catenin was a predictor of decreased survival of borderline statistical significance (Table 2), after adjusting for age, tumour grade, stage and p53 status.

When progression-free survival was evaluated, the same pattern was found. Loss of expression of E-cadherin was significantly associated with tumour progression in

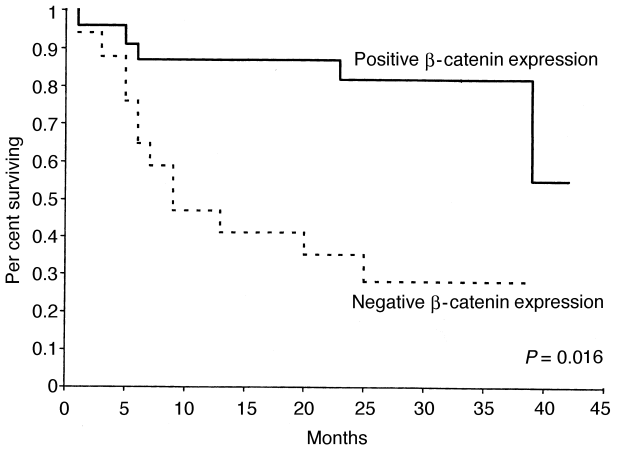

Fig. 3. Survival distribution in patients with bladder cancer according to β-catenin expression status.

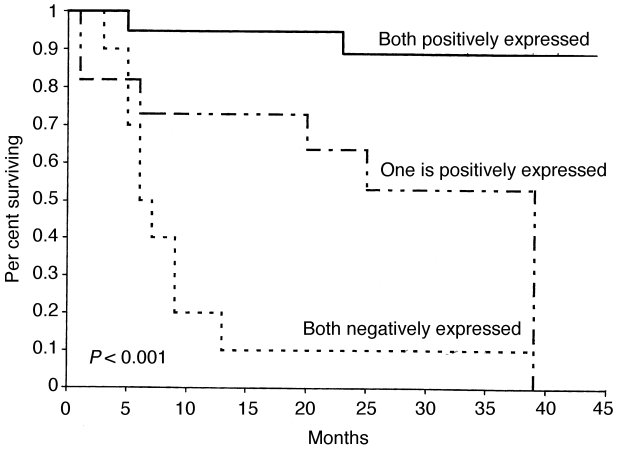

Fig. 4. Survival distribution in patients with bladder cancer according to E-cadherin and β-catenin expression status.

the univariate (HR = 10.21 [95% CI, 3.13–33.31]) and multivariate analyses (HR = 5.17 [95% CI, 1.13–23.53]). In contrast, β-catenin did not show any correlation with progression-free survival in the multivariate analysis (HR = 2.28 [95% CI, 0.64–8.06]). The association of loss of expression of both E-cadherin and β-catenin was also a marginally significant independent predictor of progression-free survival (HR = 8.04 [95% CI, 0.99–64.78]) in the multivariate analysis.

4. Discussion

E-cadherin is a calcium-dependent transmembrane glycoprotein mediating cell–cell adhesion in epithelial tissues. Tumour invasion of the surrounding tissues by cancerous cells and their metastatic spread requires their detachment from the primary lesion, which can be favoured by the reduction of membranous E-cadherin [4,5]. A decreased immunoreactivity of E-cadherin has been observed in a variety of carcinomas, usually associated with advanced stage and progression [6]. Several studies have examined the role of E-cadherin in transitional bladder carcinoma, showing a high incidence of alterations in its expression [7–12]. These studies have uniformly revealed clear associations of decreased E-cadherin expression, with high grade and advanced stage tumours. Decreased E-cadherin expression has also been correlated with poor survival [7] and recurrence [9]. These findings have been supported by additional studies in bladder [8,10,11,13,14] and upper urinary tract [15] transitional cell carcinomas.

Our study confirms these data, showing a significant prognostic impact of E-cadherin expression in patients with carcinoma of the bladder. Loss of E-cadherin expression was associated with high grade and invasive stage. Moreover, loss of E-cadherin expression was a significant prognostic indicator of decreased survival, independent of known prognostic factors such as grade, invasiveness and p53 status, and of reduced progression-free survival, in the multivariate analysis. These findings suggest that the expression of E-cadherin might be a useful prognostic marker for the clinical assessment of bladder cancer, independent of and, consequently, complementary to other established prognostic factors. This prognostic effect seems to be particularly accentuated in patients with invasive bladder cancer. In these patients, the detection of E-cadherin loss might be useful in selecting those patients who would benefit most from early aggressive therapy. Nevertheless, this should be interpreted cautiously and needs to be confirmed in large-scale studies.

β-catenin is an E-cadherin-associated protein that links the cytoplasmic tail of E-cadherin to the actin cytoskeleton of the cell, and is necessary for E-cadherin function [16]. In one study, decreased β-catenin immunoreactivity was associated with poor outcome in bladder cancer [17]. Two recent studies explored the prognostic value of alpha-, beta- and gamma-catenin expression in bladder cancer. Their results suggested that alpha-catenin [18] and gamma-catenin [19] expression correlated with prognosis. It must be taken into account that immunodetection of a given protein does not necessarily mean that is fuctional. The results of our study showed a significant association between loss of β-catenin expression and grade, stage, tumour progression and decreased survival. However, when a multivariate analysis was done, their prognostic effect appeared to be of limited value. Interestingly, when expression of β-catenin and E-cadherin was analysed, simultaneous loss of expression for the two proteins was a strong and marginally significant predictor for poor survival.

CD44 is a cell surface glycoprotein implicated in extracellular matrix adhesion and cellular migration. Primary transcripts of the CD44 gene can be alternatively spliced to produce a variety of messenger RNA species. Variant mRNA contain sequences from one or more additional exons (v1–10) [20]. Expression of CD44 variants has been associated with progression in colorectal cancer and other carcinomas [21]. Several studies have shown an altered expression pattern in transitional cell carcinoma, without a clear prognostic effect [22–24]. In our study, there was no association between variant CD44 expression and clinico-pathological variables or prognosis. Recent reports suggest that CD44v8–10 expression, detected by competitive reverse transcription–polymerase chain reaction analysis, may be useful in early diagnosis of bladder cancer [25].

In conclusion, the results of this study suggest that loss of E-cadherin, and to a lesser extent, loss of β-catenin, as determined by immunohistochemistry, are important prognostic markers in patients with bladder carcinoma. Furthermore, the combined assessment of both E-cadherin and β-catenin immunoreactivities appears to improve its prognostic value. These effects appear to be independent of other established prognostic factors used in clinical practice. Thus, assessment of E-cadherin and β-catenin expression may predict the potential behaviour of bladder tumours, and accordingly dictate appropriate therapeutic strategies. However, this retrospective study, with a limited number of cases, does not allow definite conclusions to be drawn, and the results should be cautiously interpreted. Large, prospective and well designed clinico-pathological studies to validate the prognostic value of E-cadherin and β-catenin immunoreactivity should be performed.

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