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Expression of E-cadherin, β-catenin, and epithelial membrane antigen does not predict survival in patients with high-risk non-muscle-invasive bladder cancer

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Abstract

The aim of the study: *was to validate the value of E-cadherin and β-catenin expression and to test an alternative prognostic marker, epithelial membrane antigen (EMA).*

Material and methods: *Forty-nine consecutive patients with primary stage T1 non-muscle-invasive bladder cancer (NMIBC) were enrolled in this study. Tissue specimens were stained with the following mouse anti-human antibodies: anti-E-cadherin, anti-β-catenin, and anti-EMA. Reaction intensity within cancer cells was assessed according to the immunoreactive score (IRS). Finally, the association between the expression of selected proteins and patient survival was assessed.*

Results: *The mean follow-up was 34.8 months. Recurrence-free survival, progression-free survival, and overall survival (OS) were 47.5%, 72.5%, and 72.5%, respectively. Differences in the IRS for β-catenin and EMA were found clinically, but were not statistically significant in prediction of the risk of disease progression ($p > 0.05$). No difference in protein expression was observed regarding the risk of recurrence, OS, or cancer-specific mortality ($p > 0.05$). Stratification of patients based on the IRS into three groups (poor, moderate, and intensive reaction) failed to identify a prognostic marker among the tested proteins ($p > 0.05$).*

Conclusions: *Expression of E-cadherin, β-catenin, and EMA cannot reliably predict survival in patients with high-risk NMIBC. Further searches are needed to identify tissue markers of progression and recurrence in NMIBC.*

Key words: *bladder cancer, disease progression, immunohistochemistry, recurrence, survival.*

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Introduction

Bladder cancer is the most common malignancy within the urinary tract. In most cases, the disease is diagnosed at the non-muscle invasive stage, which potentially can be treated with bladder preservation. However, after transurethral resection of the tumour (TURBT), there is a significant risk of disease recurrence and progression. This is particularly relevant in stage T1, high-risk cases. Progression of non-muscle-invasive bladder cancer (NMIBC) into muscle-invasive disease (MIBC) is associated with a relatively poor prognosis and shorter survival compared with cases that were initially MIBC [1]. For this reason, the early identification of patients with T1 bladder cancer who are at the highest risk of progression may drive clinical decisions, lead to immediate qualification for radical surgery, and improve oncological outcomes.

Stratification of the risk of bladder cancer progression after TURBT was initially based on clinical and pathological factors included in the European Organisation for Research and Treatment of Cancer (EORTC) and Club Urologico Espanol de Tratamiento Oncologico (CUETO) tables [2, 3]. From a clinical point of view, these

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tools are simple and reproducible; however, their accuracy is not perfect. Apart from cases of extensive high-grade T1 tumours with concomitant foci of carcinoma *in situ* (CIS), it is difficult, using these tools, to identify patients at the highest risk of progression who would benefit from immediate cystectomy. Consequently, a search for new predictive tools is ongoing. Among potential candidates, immunohistochemical assessment of the expression of crucial cancer proteins seems very attractive as it is a widely available, relatively inexpensive, and reproducible method. Many tissue markers of bladder cancer have already been proposed, including proliferation markers (e.g. Ki-67, MIB1), suppressor gene products (e.g. p53), growth factors (e.g. FGFR3), oncogenes (e.g. cyclin D1 and D3), and many others. Recently, numerous research groups have published clinically sound, but inconsistent results on the value of E-cadherin and β-catenin as prognostic markers. However, to date, no single marker has been recognised as a reliable and clinically useful predictor of survival in patients with NMIBC.

The aim of this study was to objectively validate the usefulness of E-cadherin and β-catenin expression as prognostic markers and to test an alternative protein marker, epithelial membrane antigen (EMA), in patients with high-risk T1 bladder cancer.

Material and methods

Forty-nine consecutive patients with primary high grade T1 conventional urothelial bladder carcinoma were enrolled in this observational study. The mean age of the cohort was 70.6 years, and the male to female ratio was 3.6 : 1. All patients underwent both primary and restaging TURBT. Exclusion criteria were as follows: any TURBT performed in the past, concomitant CIS, lack of a detrusor muscle layer in the specimen (stage Tx), a history of systemic treatment for any cancer within the last 10 years, and lack of at least a 6-month follow-up with at least one cystoscopy done.

Tissues were collected from all participating patients as part of routine clinical care at the time of TURBT. After initiation of the study, microscopic slides of all patients were re-evaluated by an experienced uropathologist. Afterwards, corresponding archival paraffin blocks were serially cut into 3-μm slices with a microtome for immunohistochemical staining. Antigen retrieval was performed by 20-minute thermal incubation in Target Retrieval Solution (Dako, Denmark) in all cases. Staining was performed in an automatic station (Dako, Denmark). The following primary antibodies were used: mouse anti-human E-cadherin (clone NCH38, Dako IS059, Denmark), mouse anti-human β-catenin (clone β-catenin 1, Dako IS702, Denmark), and mouse anti-human EMA (clone E29, Dako I629, Denmark). Only ready to use, autostainer-dedicated reagents were used.

For an objective assessment of the immunohistochemical reaction intensities, we adopted the immunoreactive score (IRS) scale according to Remmele and Stagner. This is a semi-quantitative scale incorporating the percentage of positive cells and staining intensity in five visual fields of the light microscope at 200× magnification. The final IRS is a product of the percent positive cells (0, no cells with positive reaction; 1, ≤ 10% cells with positive reaction; 2, 11% to 50% cells with positive reaction; 3, 51% to 80% cells with positive reaction; 4, > 80% cells with positive reaction) and staining intensity (0, no colour reaction; 1, poor colour reaction; 2, moderate colour reaction; 3, intensive colour reaction). It can range from 0 to 12 (0-2, poor reaction; 3-5, moderate reaction; 6-12, intensive reaction). Five visual fields for IRS calculation were randomly chosen in each patient and the mean value from three calculations was recorded. Study endpoints were as follows: overall mortality, cancer-specific mortality, freedom from recurrence, and freedom from progression. All the endpoints were assessed with regard to the IRS. The Shapiro-Wilk test confirmed the normal distribution of all variables. Levene's test was applied for the assessment of the equality of variances. Results in subgroups were compared with the unpaired t-test and Pearson test for quantitative and ordinal variables. The differences were considered statistically significant when the p-value was < 0.05. This is a retrospective and non-interventional study; hence, approval of the institutional review board was waived. All patients provided written consent to participate in the study.

Results

The final per protocol analysis was based on 40 patients, including nine women. From the initial study group, microscopic slides were not available for five patients and bladder cancer was downstaged to stage Ta disease after pathological re-evaluation in four patients. The mean follow-up was 34.8 months (range 3-87 months). Adjuvant intravesical Bacillus Calmette-Guerin (BCG) therapy was implemented in 45% of patients. During the follow-up, in 21 patients recurrence occurred (52.5%), in 11 patients the disease progressed (27.5%), and 11 patients died (27.5%), including eight patients who died from bladder cancer (20%). In three patients (7.5%), a radical cystectomy was eventually performed due to disease progression or BCG failure.

The mean IRS for E-cadherin, β-catenin, and EMA were 8.28 ±3.84 (95% CI: 7.06-9.51), 8.60 ±4 (95% CI: 7.32-9.87), and 7.60 ±4.15 (95% CI: 6.28-8.93), respectively. Examples of the immunohistochemical reactions are shown in Figure 1. Clinically significant differences in the IRS were observed for β-catenin and EMA regarding the risk of disease progression; however, they did not reach statistical significance (Table 1). The percentages of cases with an abnormal reaction for E-cadherin (poor and

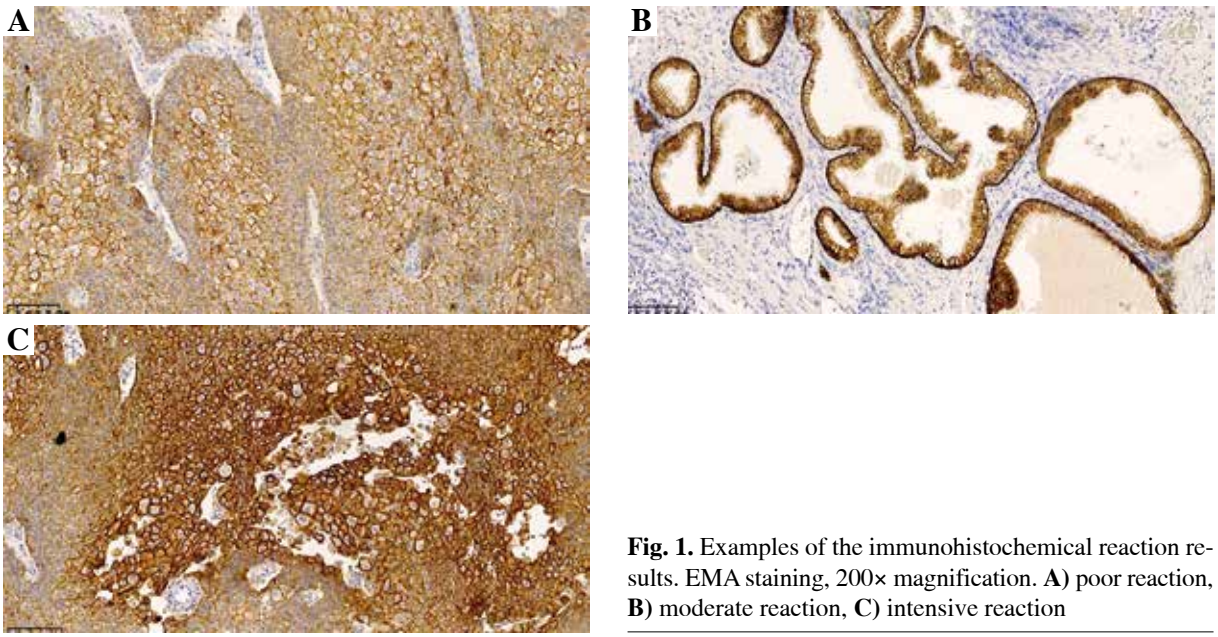

Fig. 1. Examples of the immunohistochemical reaction results. EMA staining, 200× magnification. **A)** poor reaction, **B)** moderate reaction, **C)** intensive reaction

Table 1. Mean immunoreactive score (IRS) scale values for the examined proteins regarding mortality, recurrence, and progression of bladder cancer

	E-cadherin	β-catenin	Epithelial membrane antigen
All-cause mortality			
Alive	8.34 ±3.67	8.50 ±4.20	7.67 ±4.15
Dead	8.14 ±4.43	8.84 ±3.59	7.40 ±4.34
p	0.88	0.82	0.85
Cancer-specific mortality			
Dead from bladder cancer	7.69 ±5.07	8.25 ±4.03	6.68 ±4.82
Dead from other causes	9.33 ±2.31	10.4 ±1.6	9.33 ±2.31
p	0.61	0.40	0.39
Freedom from recurrence			
Recurrence	8.61 ±3.86	8.67 ±4.37	7.01 ±4.60
No recurrence	7.92 ±3.89	8.52 ±3.66	8.25 ±3.59
p	0.57	0.91	0.35
Freedom from progression			
Progression	7.64 ±4.61	7.36 ±4.63	5.85 ±4.76
No progression	8.53 ±3.57	9.06 ±3.71	8.26 ±3.77
p	0.52	0.23	0.10

moderate reaction intensity) were 36.4% and 27.6% among patients with and without disease progression, respectively ($p = 0.59$). No difference in protein expression was observed regarding the risk of recurrence, overall survival (OS), and cancer-specific mortality (Table 1). Also, stratification of patients based on the IRS into three groups (poor, moderate, and intensive reaction) failed to identify a prognostic marker among the tested proteins (Table 2). Finally, there were no differences between male and female patients regarding all study end-points and the IRS ($p > 0.05$).

Discussion

Up to 45% of patients with primary T1 urothelial bladder carcinoma eventually progress to MIBC during the follow-up after TURBT [2]. Their identification is of ut-

Table 2. Reaction intensity based on the immunoreactive score (IRS) scale for E-cadherin, β-catenin, and epithelial membrane antigen (EMA) regarding the recurrence and progression of bladder cancer

Reaction intensity		Total (%)	Recurrence			Progression		
			Recurrence (%)	No recurrence (%)	*p*	Progression (%)	No progression (%)	*p*
E-cadherin	Poor	10	9.5	10.5	0.61	18.2	6.9	0.57
	Moderate	20	14.3	26.3		18.2	20.7	
	Intensive	70	76.2	63.2		63.6	72.4	
β-catenin	Poor	12.5	14.3	10.5	0.80	18.2	10.3	0.59
	Moderate	12.5	9.5	15.8		18.2	10.3	
	Intensive	75	76.2	73.7		63.6	79.3	
EMA	Poor	15	23.8	5.3	0.16	27.3	10.3	0.16
	Moderate	25	28.6	21		36.4	20.7	
	Intensive	60	47.6	73.7		36.4	69	

most importance, as the cancer-specific survival (CSS) in this particular group of patients does not exceed 35% [1]. On the other hand, the 5-year CSS in patients undergoing radical cystectomy due to NMIBC exceeds 80% [4-6]. We examined expression of three proteins in a cohort of patients with primary T1 bladder cancer: the cell adhesion-associated proteins, E-cadherin and β-catenin, and the epithelial-specific glycoprotein, EMA. Our results support the criticism regarding E-cadherin and β-catenin expression as prognostic tools and did not identify a novel tissue marker. This is the first immunohistochemical study to test the prognostic significance of protein markers in patients with bladder cancer by objective quantification of their expression.

E-cadherin belongs to a family of transmembrane glycoproteins, responsible for cell adhesion to other cells or the extracellular matrix [7]. Its expression is observed in 88% of normal human bladders, while, during malignant transformation, it decreases proportionally with the tumour invasiveness [8]. The crucial role of decreased E-cadherin function during carcinogenesis is epithelial-mesenchymal transition, which is responsible for the invasion into the lamina propria in T1 bladder tumours [9].

A few research groups have already reported on the prognostic significance of E-cadherin expression in patients with NMIBC. Erdemir *et al.* found that low expression of E-cadherin is associated with greater risk of recurrence of high-grade T1 tumours [10]. Khorrami *et al.* confirmed this finding in a group of patients with low-grade NMIBC [11]. These results were further supported by Muramaki *et al.* and Liu *et al.*, who additionally indicated that loss of function of E-cadherin may be as important as the gain of function of N-cadherin [12, 13]. Finally, Mahnken *et al.* observed an inverse correlation between E-cadherin expression and the risk of T1 bladder cancer recurrence, as well as the T1 substage and p53 index [14]. In contrast, in our study, expression of E-cadherin was not predictive for NMIBC recurrence. One potential reason for the observed discrepancy may be the longer follow-up period in our study, as the majority of low-risk NMIBCs recur early. Raspollini *et al.* and Zhao *et al.* independently published similar negative results [15, 16]. The majority of above-mentioned studies were limited by their inability to assess the impact of E-cadherin expression on tumour progression, which seems to be the most important factor affecting the discrepancies. This may have been due to heterogeneous study populations, short or unspecified follow-up, and low rates of progression.

The effect of E-cadherin expression on the progression of high-risk NMIBC and patient survival remains controversial. On one hand, an increased risk of NMIBC progression in patients with decreased E-cadherin expression was recently reported by Breyer *et al.* in a group of Ta tumours and by Raspollini *et al.* in a group of T1 tumours [15, 17]. On the other hand, Zhao *et al.* reported no effect of E-cadherin expression on the risk of T1 bladder cancer progression or on patient survival [16]. As E-cadherin seems to be one of the most promising tissue markers of progression in NMIBC patients, validation studies were urgently needed. For this reason, we decided to evaluate this protein in our cohort of patients. Similar to the findings of Zhao *et al.*, we found no effect of E-cadherin expression on patient survival. Moreover, the reliability of previously published studies is limited by the lack of a standardised assessment of immunohistochemical reaction intensity. Our study failed to confirm the prognostic significance of E-cadherin expression using a fully objective quantitative histological method and adequately long clinical follow-up of patients. We adopted the IRS, a precise 13-level scale of reaction intensity, based on the percentage of positive cells and staining intensity.

β-catenin is part of the catenin family of cell adhesion associated proteins. β-catenin forms a bridge between

E-cadherin and α-catenin, ensuring binding of E-cadherin with the cytoskeleton [18]. Similar to E-cadherin, expression of β-catenin in urothelial cancer is decreased and inversely correlated with the tumour stage and grade [19]. The catenin family has been extensively studied in bladder cancer. Similar to cadherins, published results are inconclusive. In the first study by Shimazui *et al.*, the predictive value of catenin expression was confirmed only for MIBC and not for NMIBC cases [20]. In contrast, Schrier *et al.* reported that the expression of α-catenin can predict the progression of NMIBC [21], while Senol *et al.* found a correlation between β-catenin expression and the risk of recurrence and progression of NMIBC [22]. Finally, Clairotte *et al.* reported on the predictive value of γ-catenin and not of α-catenin [23], while Kashibushi *et al.* and Reis *et al.* independently did not find any prognostic significance of α-, β-, or γ-catenin in patients with urothelial cancer within the upper urinary tract [24, 25]. Moreover, it remains unknown whether low or high expression, if any, could predict the recurrence or progression of NMIBC.

All these conflicting findings regarding the prognostic value of catenin expression in patients with NMIBC led us to examine β-catenin in our study. We did not observe a statistically significant correlation between its expression and survival, although the expression was noticeably lower in patients whose disease progressed. In view of our and previously published results, we think catenins are more controversial than practical as prognostic markers in bladder cancer.

The cell surface mucin glycoprotein EMA is expressed by epithelial-origin cells. Normal function of the protein is protective and regulatory. It acts as a barrier on the apical surface of epithelial cells [26]. In normal urothelium, only the upper layer cells express EMA, while in urothelial cancer, EMA is also expressed in deeper cell layers [27]. Chaotic and irregular expression of EMA is a characteristic of high-grade tumours [28].

It was postulated that the expression or concentration of EMA in urine could be used as a non-invasive diagnostic test for bladder cancer [29]. In contrast to conventional urine cytology, EMA testing can detect up to 50% of grade 1 urothelial carcinomas [30]. Recently, Attallah *et al.* outlined the higher sensitivity of combined nuclear matrix protein 22 (NMP22) and EMA testing for bladder cancer detection compared with both tests alone [31]. However, this application has never been widely accepted.

In the present study, we observed decreased expression of EMA in patients with unfavourable results at follow-up, including recurrence, progression, and death. The most important difference was found between patients with and without progression. However, none of our observations reached statistical significance. Interestingly, while our results confirmed that EMA can be regarded as a marker of bladder carcinoma, in high-grade and high-risk cases, low expression of the protein is more typical than high expression. The only study that tested EMA as a prognostic marker in patients with histologically confirmed bladder cancer focused on localisation of a positive reaction. Takashi *et al.* found that stromal expression is a prognostic factor of poor survival [27]. To the best of our knowledge, no other study has reported on the prognostic value of EMA expression.

The discovery of a marker that can identify, early on, patients with NMIBC who will progress during follow-up would be useful for recognising those who should undergo immediate cystectomy. Our study failed to identify such a marker; furthermore, it raises questions about the clinical utility of E-cadherin and β-catenin expression. Finally, it should be highlighted that the timing of cystectomy in NMIBC patients is also controversial. It can be considered directly after the initial diagnosis or after the first- or second-line intravesical therapy. Studies reported that the survival of NMIBC patients depended on the timing of surgery, suggesting that immediate cystectomy is beneficial [4, 32]; however, some long-term observational studies reported that the CSS after TURBT and BCG therapy is similar to that of immediate cystectomy [33, 34]. All these studies were retrospective and presented historical cohorts.

As discussed above, studies with similar objectives have been published previously. However, the clear majority of them suffered from significant limitations. In our study, we used an objective quantification of protein expression, so the risk of biased data was significantly reduced. In addition, reliable conclusions could be made because the length of follow-up was long enough to diagnose the cases of disease progression, which usually occurs between the first and fifth year after surgery. Finally, the pathological work-up was standardised and all microscopic slides were reassessed by an experienced uropathologist to exclude the risk of over- or understaging, which is a known phenomenon in bladder cancer pathology. The most important limitation was the low number of patients included. We cannot rule out that inclusion of more patients would bring statistically significant findings, especially for EMA. Moreover, urothelial carcinoma has an inhomogeneous histological appearance, including in terms of immunohistochemistry. Although we assessed five randomly chosen visual fields, there is still a risk of bias.

Conclusions

It is highly questionable whether expression of E-cadherin or β-catenin can reliably predict the recurrence or progression of high-risk NMIBC. Also, EMA cannot be used as prognostic marker in T1 bladder cancer. Thus, tissue markers of progression and recurrence are still needed for patients with high-risk NMIBC.

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