

# The cadherin switch assessment in the epithelial-mesenchymal transition of urothelial bladder carcinomas

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## Abstract

**Introduction:** The epithelial-mesenchymal transition (EMT) process is a complex molecular mechanism that is involved in the acquisition of an aggressive, invasive and metastatic phenotype by carcinomas. The cadherin switch consists in the alteration of E-cadherin and N-cadherin expression and is specific for the EMT process. **Materials and Methods:** This study included 35 cases of primitive urothelial carcinomas investigated in relation with clinicopathological prognostic parameters and expression of E- and N-cadherins in the advancing edge and intratumoral compartments. **Results:** In both compartments, the immunoeexpression of E-cadherin decreased, while that of N-cadherin increased in high grade, deeply invasive, or those cases with lymph node metastases and advanced stages carcinomas, with a negative linear correlation observed between their expression percentage values. In this study, it was observed the presence of cadherin switch in urothelial carcinomas, the variation of the two proteins' immunostaining patterns being higher at the advancing edge. The presence of N-cadherin in intratumoral compartment designated it as actively involved in EMT process. **Conclusions:** The analysis of cadherins switch can be used to identify superficial urothelial carcinoma with invasion and metastasis potential.

**Keywords:** urothelial carcinoma, cadherin switch, epithelial-mesenchymal transition.

## Introduction

Bladder cancer is one of the most common malignant cancers, ranking as the seventh type of cancer among men, with a worldwide incidence and mortality, which has been relatively constant in the last 10 years [1–3]. Most lesions are urothelial carcinomas, the assessment of progression and relapse risks being sometimes real challenges and the subject of numerous research reports. In this context, urothelial carcinomas stratification through the biomolecular mechanisms involved in the initiation, development and tumor progression may lead to the identification of aggressive lesions and associated proteins that can be used as therapeutic targets for prognosis improvement.

One of the mechanisms extensively studied in the recent years is the epithelial-mesenchymal transition (EMT), which refers to the epithelium plasticity [4]. EMT process consists of the appearance of cells with mesenchymal phenotype in the epithelial tissues as a result of polarity and interepithelial junctions alteration, as well as a reorganization of the cytoskeleton and organelles redistribution [5, 6].

Initially designated as a molecular mechanism involved in embryogenesis and tissue healing, it was found afterwards that EMT process is involved in tumor progression,

respectively in invasion and metastasis of cancers with various locations [7, 8].

There are relatively few studies that have examined the markers associated with EMT process for bladder urothelial carcinomas [9]. Moreover, the EMT process should be analyzed in relation to tumor compartments (intratumoral vs. advancing edge), the advancing edge being considered the active compartment, in which the tumor cells begin to express mesenchymal markers, lose the intercellular adhesion and acquires migration properties [5, 10].

Among the markers associated with EMT process are the mesenchymal (vimentin), epithelial (cadherin, cytokeratin) and transcription factors (Snail, Slug, Twist) [11].

In this study, we analyzed the E-cadherin and N-cadherin comparative immunoeexpression in urothelial bladder carcinomas, in relation with pathological prognostic parameters and for cadherins switch characterization, which is specific for EMT process.

## Materials and Methods

The study included 35 cases of urothelial bladder carcinomas from patients hospitalized in the Department of Urology from the Emergency County Hospital of

Craiova, Romania, between 2010–2015, and diagnosed in the Laboratory of Pathology of the same Hospital. Biological material was represented by total cystectomy specimens, which were fixed in 10% buffered neutral formalin, processed by paraffin embedding and Hematoxylin–Eosin (HE) staining.

We investigated clinical and pathological parameters as age, gender, degree of differentiation, depth of invasion (pT), lymph node status (pN) and tumoral stage. For the assessment of the lesions, we used the *World Health Organization* (WHO) 2004 staging system [12]. In this study were included only primary urothelial carcinomas which had no distant metastases and without any pre-operative chemotherapy or radiotherapy.

For immunohistochemical analysis, we used mouse antihuman monoclonal antibodies (Table 1).

**Table 1 – The antibodies panel**

Antibody	Clone	Dilution	Pretreatment	External positive control
<i>E-cadherin</i>	NCH38/DAKO	1:100	Microwaving in citrate buffer, pH 6	Mammary gland
<i>N-cadherin</i>	6G11/DAKO	1:40	Microwaving in citrate buffer, pH 6	Tonsil

The sections were incubated overnight at 4°C with primary antibodies after antigen retrieval in citrate buffer pH 6, endogenous enzyme blocking, and unspecific blocking. For visualization, it was used the LSAB2 system (Dako, Redox, Romania, code K0675) and as detection chromogen 3,3'-diaminobenzidine tetrahydrochloride (Dako, Redox, Romania, code K3468). We used external positive controls and respectively negative controls, by omitting the primary antibody.

The immunohistochemical analysis was done intra-tumorally and at the advancing edge of the tumors. Immunohistochemical quantification took into account the number of labeled cells and the immunostaining intensity, leading to a final expression score by multiplying the score values assessed for these two parameters [13]. Thus, according to the number of labeled tumor cells, the cases were distributed according to the following scale:

(1) <25% labeled cells, (2) 26–49% labeled cells, and (3) >50% labeled cells. Depending on staining intensity, the scale was the following: (1) poor, (2) moderate, and (3) strong.

For the statistical analysis, the immunoexpression was considered “low” for 1–4 score and “high” if the score was 6–9. Further, the mean value of the expression score was determined for each of the studied parameters and variables. These values were also distributed on a scale as follows: (1) low for values <4, (2) moderate for values >4 and <6, and (3) high for values >6.

Student's *t*-test, chi-square ( $\chi^2$ ) and Pearson's correlation test, under the SPSS 10 software were used for the statistical validation of data. Results were considered significant for *p*-values <0.05.

Image acquisition was performed using a Nikon Eclipse E600 microscope and the Lucia 5 software.

The study was approved by the local ethical committee, and written informed consent was obtained from all the patients.

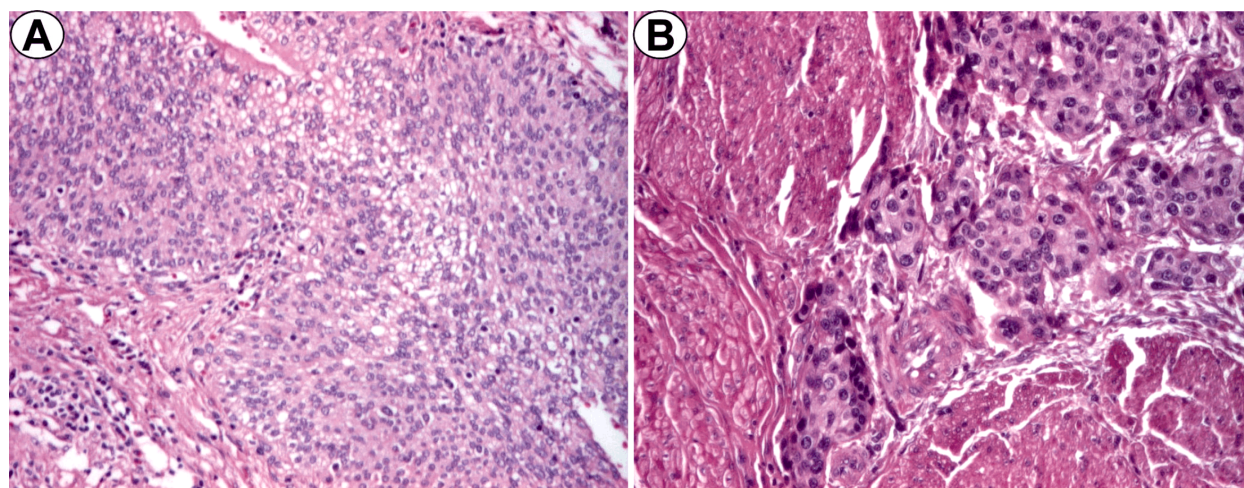
## Results

The analysis of the clinicopathological data indicated an average diagnosis age of 62.4 years, in 88.6% of cases involving patients aged over 50 years (Table 2). The majority of cases were male patients (80%) and the ratio male/female was 4. Most investigated lesions were low-grade carcinomas (60%), with superficial invasion in lamina propria (45.8%), without lymph node metastasis (94.3%) and in early stages (I/II 51.4%) (Table 2, Figure 1).

**Table 2 – Clinical and pathological parameters**

Parameter	Variable (No. of cases)
Age	<50 years: 4; >50 years: 31
Gender	Males: 28; Females: 7
Differentiation degree	LG: 21; HG: 14
Depth of invasion (T)	T1: 16; T2: 13; T3: 4; T4: 2
Lymph node status (N)	N0: 33; N1: 2
Stage	I: 16; II: 12; III: 4; IV: 3

LG: Low grade; HG: High grade.



**Figure 1 – Urothelial carcinomas (HE staining, ×100): (A) Low grade, lamina propria invasion; (B) High grade, muscularis propria invasion.**



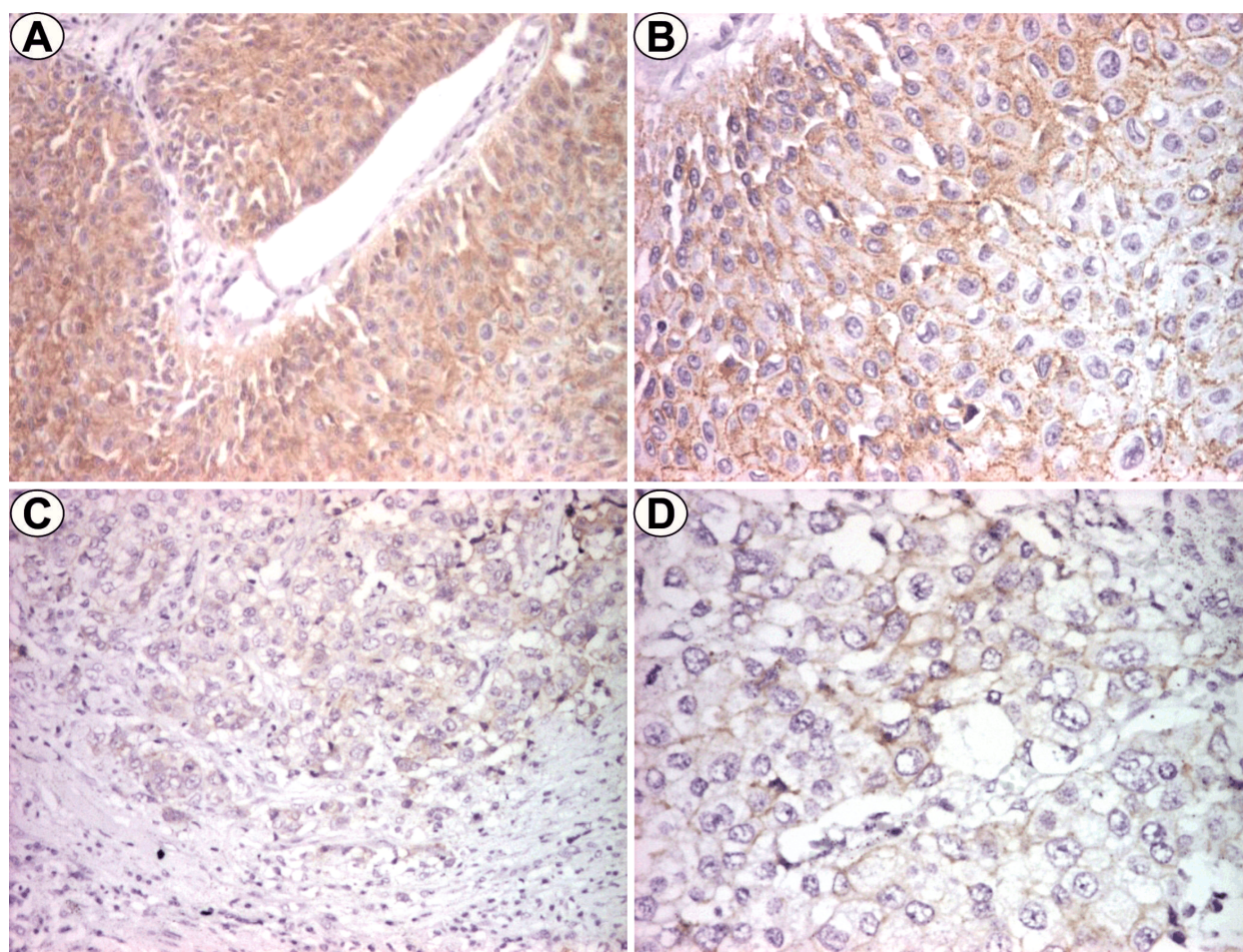
### E-cadherin expression

Immunohistochemical analysis indicated the presence of tumor cells membrane E-cadherin staining in majority of analyzed cases.

Independently of the clinicopathological parameters, the E-cadherin reaction was significantly higher intratumorally, where the average percentage of labeled cells was around 41% ( $40.9 \pm 18.9$ ), with a moderate mean value of the expression score of 4.5, whereas at the advancing edge the percentage of labeled cells was of around 31%

( $30.8 \pm 18.9$ ) and with a low mean value of the expression score of 2.9. The differences were significant also from statistical point of view. Student's *t*-test applied for the labeling index had a *p* value of 0.014 and  $\chi^2$ -test applied for mean value of the expression score had a *p* value of 0.002.

The E-cadherin immunoexpression in relation to the tumors **degree of differentiation** indicated higher values in the case of low-grade compared with high-grade carcinomas (Figure 2).



**Figure 2 – E-cadherin immunostaining:** (A) Low grade,  $\times 100$ ; (B) Low grade,  $\times 200$ ; (C) High grade,  $\times 100$ ; (D) High grade,  $\times 200$ .

Thus, in the intratumoral compartment, the intensity level of reactions was variable. In low-grade tumors, the mean percentage of labeled cells was around 50%, with a moderate mean value of the expression score, whereas in high-grade lesions the reaction presented low/moderate intensity mean percentage of labeled cells of around 25%, and a low mean value of the expression score. The difference was significant also from statistical point of view (Table 3, Figure 3a).

At the advancing edge, the intensity expression of E-cadherin in low-grade carcinomas was variable with a mean percentage of labeled cells of around 37% and a low mean value of the expression score whereas in high-grade lesions the reaction had low intensity, with a mean percentage of labeled cells of around 20% and a lower mean value of the expression score, the difference being

statistically significant as in the case of intratumoral compartment (Table 3, Figure 3b).

The analysis of E-cadherin immunoreaction in relation to the **invasion depth** indicated statistically significant differences both intratumorally and at the advancing edge. Superficial invasive carcinomas (in the lamina propria – pT1) presented values of labeling index and mean values of the expression score higher than deeply invasive lesions (pT2–pT4) (Table 3, Figure 4).

Thus, in the case of superficial urothelial carcinomas (pT1), the reaction intensity to the anti-E-cadherin antibody was variable, with more than 52% labeled cells and a high mean value of the expression score at intratumoral level. In turn, at the advancing edge, the labeling index was only of 40% with a low to moderate high mean value of the expression score.

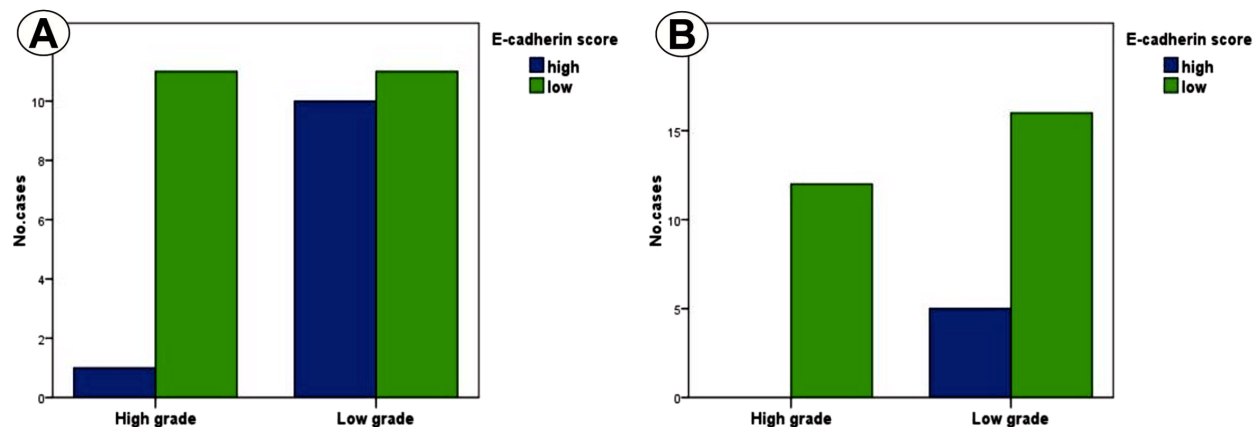
By contrary, in case of carcinomas invading at least muscularis propria (pT2–T4), E-cadherin reactions presented low to moderate intensity, with around 30% labeled cells

and a low mean value of the expression score at intratumoral level, respectively 22% and even lower mean value of the expression score at the advancing edge (Table 3).

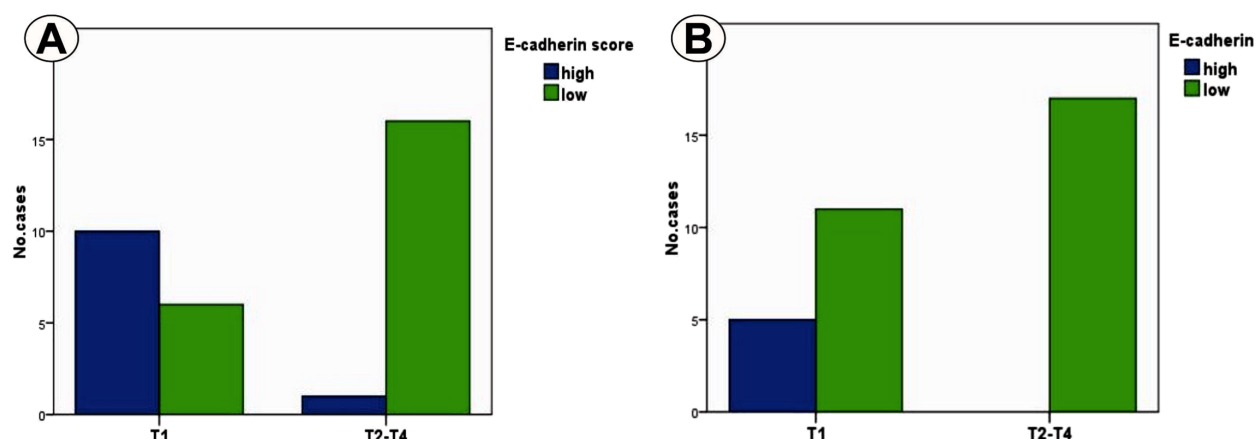
**Table 3 – Distribution of E-cadherin and N-cadherin scores in relation to the analyzed parameters of studied urothelial carcinomas**

Parameter	Variable	E-cadherin				N-cadherin			
		IT		AE		IT		AE	
		ESMV	p*	ESMV	p*	ESMV	p*	ESMV	p*
Age	<50 years	4.4	0.199	2.8	0.357	6.0	0.255	6.0	0.189
	>50 years	5.3		4.6		5.1		5.3	
Gender	Male	4.4	0.338	3.0	0.252	4.6	0.211	5.3	0.172
	Female	5.0		2.6		4.0		5.2	
Differentiation degree	LG	5.8	0.021	3.9	0.047	2.5	0.182	4.0	0.086
	HG	2.2		1.2		4.7		5.5	
Lymph node (pN)	N0	4.7	0.302	3.0	0.538	3.8	0.086	4.8	0.282
	N1	1.0		1.0		9.0		9.0	
Depth of invasion (pT)/Stage	T1	6.1	0.001	4.0	0.012	3.3	<0.0001	5.3	0.006
	T2	3.3		2.1		2.8		4.0	
	T3	3.4		2.0		6.0		6.0	
	T4	1.0		1.0		9.0		9.0	

IT: Intratumoral; AE: Advancing edge; ESV: Mean value of the expression score; p\*: P-value of the  $\chi^2$ -test; LG: Low grade; HG: High grade.



**Figure 3 – The cases distribution in relation to tumor grade and E-cadherin score: (A) Intratumoral; (B) At the advancing edge.**



**Figure 4 – The cases distribution in relation to depth of invasion and E-cadherin score: (A) Intratumoral; (B) At the advancing edge.**

### N-cadherin expression

N-cadherin immunoexpression was identified at the cytoplasmic and membrane levels in 45.7% of investigated cases, the reaction being present also in some stromal lymphocytes and fibroblasts (Table 3, Figure 5). Among

the positive cases, 87.5% were represented by high-grade carcinomas with invasion at least in muscularis propria. Similar immunostains were found on two cases of superficial urothelial carcinomas with lamina propria invasion.

In contrast to E-cadherin, N-cadherin immunoexpression was significantly higher in the advancing



edge compartment compared to the intratumoral one. Thus, at intratumoral level, the average percentage of labeled cells was of around 33% ( $32.8 \pm 13.6$ ), with a moderate mean value of the expression score of 4.5, whereas at the advancing edge the percentage of labeled cells was of around 43% ( $43.4 \pm 18.9$ ) and with a moderate to high mean value of the expression score of 5.3. The differences were significant also from the statistical point of view. Student's *t*-test applied for the labeling index had a *p* value of 0.030 and  $\chi^2$ -test applied for mean value of the expression score had a *p* value of 0.003.

In relation to the tumor **degree of differentiation**, in both tumor compartments, the highest N-cadherin immunostains were associated with high-grade carcinomas compared with the low grade, but without statistically significance ( $\chi^2$ -test *p*-value >0.05).

The analysis of N-cadherin immunoexpression in

relation to **depth of invasion**, indicated significantly lower values in lamina propria or muscularis propria invasive carcinomas (pT1–T2), as compared to deeper invasive group (pT3–T4) (Table 3, Figure 6).

Thus, intratumoral analysis of pT1–pT2 carcinomas revealed an average percentage of cells stained with N-cadherin of around 27%, with a low mean value of the expression score whereas pT3–pT4 tumors revealed an average percentage of cells stained with N-cadherin of around 42%, with a high mean value of the expression score.

Percentage values and scores for the advancing edge were of around 40% with a low mean value of the expression score for pT1–pT2 lesions, and of 50% with a high mean value of the expression score for pT3–pT4 lesions (Table 3, Figure 6).

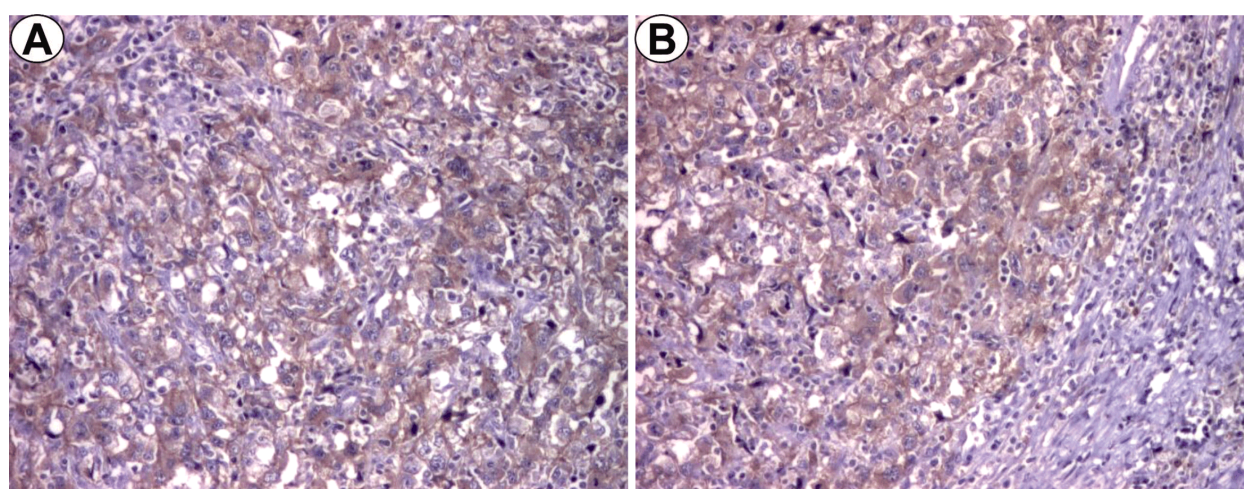


Figure 5 – N-cadherin immunostaining: (A) Intratumoral compartment,  $\times 100$ ; (B) Advancing edge,  $\times 100$ .

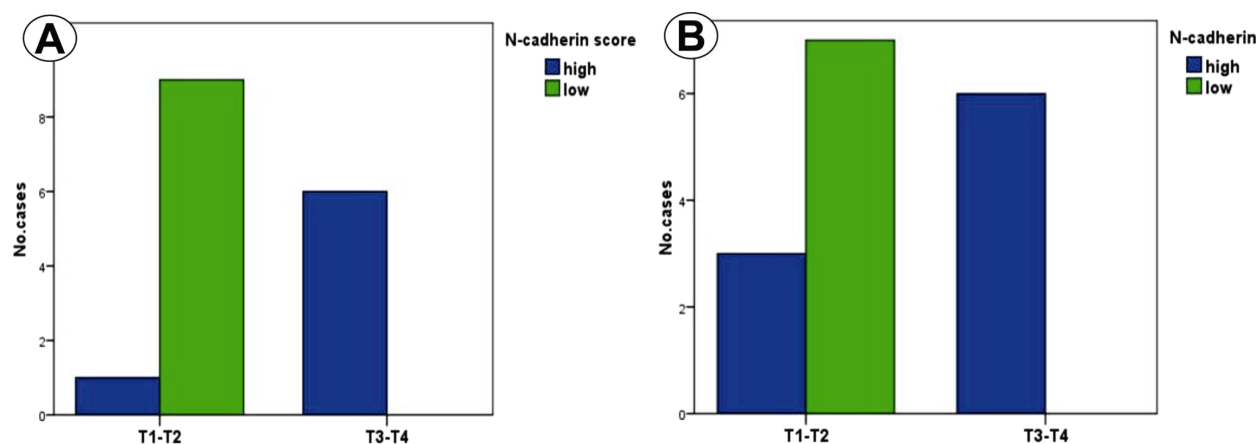


Figure 6 – The cases distribution in relation to depth of invasion and N-cadherin score: (A) Intratumoral; (B) At the advancing edge.

For the carcinomas with associated lymph node metastases (N1), the expression value of analyzed proteins was different from cases without metastasis, namely lower in the case of E-cadherin and higher for N-cadherin, but the aspects were statistically insignificant ( $\chi^2$ -test *p*-value >0.05 – Table 3). It should be noted also that the groups of analyzed lesions in relation to pT category coincide with those investigated for tumor stage, the scores and statistical results being identical for the two markers.

We noted also the absence of significant differences in the expression of E-cadherin and N-cadherin in relation to age and gender of patients ( $\chi^2$ -test *p*-value >0.05 – Table 3).

Comparative expression analysis of the two markers in terms of the percentage of labeled cells indicated a negative linear correlation, both in intratumoral and advancing edge compartments (Pearson's test *p*-value >0.0001).

## Discussion

The cadherin switch is designated as a specific process for tumoral EMT, and consists in the diminishing/loss of E-cadherin expression, associated with the appearance of N-cadherin or mesenchymal cadherin [6, 10, 14–16]. The switching process between the two cadherins is also documented during normal embryological development [17, 18].

In the numerous studies about E-cadherin expression in carcinomas with different locations, it has been found that the reduced expression is associated with aggressive lesions [19, 20]. For the bladder urothelial carcinoma, the E-cadherin expression reduction is associated with the recurrence, invasiveness, presence of metastasis and advanced tumor stage [21–23]. In our study, regardless of the clinicopathological investigated parameters, the E-cadherin immunoreactivity was significantly superior in intratumoral areas compared with advancing edge compartment ( $\chi^2$ -test  $p$ -value  $>0.05$ ); the low scores were also associated with high-grade, invasive and in advanced stages carcinomas ( $\chi^2$ -test  $p$ -value  $>0.05$ ). Although the E-cadherin values were lower in both tumor compartments in urothelial carcinoma with lymph nodes metastasis, the differences were not statistically significant ( $\chi^2$ -test  $p$ -value  $>0.05$ ).

The acquisition of the migratory mesenchymal phenotype by the tumor cells requires the repression of some epithelial genes, such as E-cadherin, resulting the loss of intercellular adhesion [10]. Some studies have indicated that among E-cadherin repressors are included transcription factors such as Snail, Slug, Zeb and Twist [24, 25]. The result of this suppression activity, along with stimulation of expression of certain mesenchymal factors were found mainly in the advancing edge of the malignant tumors, where it was observed the decreasing of E-cadherin expression and vimentin occurrence into the tumor epithelial cells [10, 26].

The mechanism is much more complex, and involves alternative molecular pathways blocking E-cadherin expression, like mitogen-activated protein kinase (MAPK), Notch, Hedgehog, Wnt and the involvement of numerous growth factors such as transforming growth factor-beta (TGF- $\beta$ 1), fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) [10, 11, 27]. These growth factors may be secreted by the tumor stromal cells, including through an autocrine stimulation of tumor cells after interaction with the tumor micro-environment [11]. Together with vimentin, other mesenchymal markers can be expressed mainly in the tumors advancing edge and are represented by matrix-metalloproteinases (MMPs) and fibronectin [28]. For examples, urothelial carcinoma of the bladder with invasion in muscularis propria, express a decreased E-cadherin/MMP9 ratio, which is an indicator of poor prognosis [6]. Some authors emphasize that in malignant tumors, within EMT process, the epithelial and mesenchymal phenotypes should be viewed as a continuum spectrum of changes, meaning the coexistence of both phenotypes within the cell population involved in tumor progression [4].

While E-cadherin is the main factor of the intercellular adhesion system, expressed in normal urothelium and low/absent in 80% of urothelial carcinomas invasive in

muscularis propria, N-cadherin is not expressed by normal urothelium, being present in 20% of superficial urothelial carcinomas and in over 50% of muscle invasive carcinomas [15, 16]. In this study, N-cadherin was expressed significantly higher at the advancing edge, compared with intratumoral compartment ( $\chi^2$ -test  $p$ -value  $>0.05$ ). N-cadherin also indicated statistically significant higher values in deeply invasive, advanced stages carcinomas ( $\chi^2$ -test  $p$ -value  $>0.05$ ). Although N-cadherin values were superior to high-grade and metastatic lesions, the results were not statistically significant ( $\chi^2$ -test  $p$ -value  $>0.05$ ). There are studies in the literature indicating the association of N-cadherin with the stage or grade of urothelial carcinomas [29, 30]. In contrast, other studies that have analyzed the expression of N-cadherin in urothelial carcinomas found no significant differences in relation with tumor stage or grade [31, 32].

N-cadherin is a transmembrane glycoprotein of the adhesion system, which is involved in the motility and cell migration during embryogenesis, but also in intercellular signaling processes, invasion, survival and metastasis of cancer cells [16, 33]. Currently, N-cadherin high expression is associated with poor prognosis for carcinomas, and the protein involvement in maintaining of an aggressive tumor behavior designates it as a possible therapeutic target [34]. N-cadherin appears to be involved in the stability of the tumor neovessels and plays a suppressor and antiproliferative role in some malignant tumors [33, 34]. N-cadherin expression is influenced by many proteins, such as growth factors (EGF), and transcription factors (Snail, Twist,  $\beta$ -catenin) [28, 33]. These complex issues can explain at least partially, the existence of some biomolecular pathways least investigated related with N-cadherin function. In this context, there are studies that found a protective effect of N-cadherin, muscle invasive N-cadherin positive urothelial carcinomas, indicating a better prognosis compared with those N-cadherin negative [30].

In our study, the switch between E- and N-cadherins was observed in both intratumoral and advancing edge regions of bladder urothelial carcinomas through the presence of negative linear correlations. The immunostaining variation for the two markers was superior to the invasion front, which is designated as the tumor compartment directly involved in the EMT process.

Nevertheless, the EMT process is relatively difficult to investigate and quantify, the main reasons including the complexity of involved biomolecular ways, the multitude of stromal elements in the advancing edge, the spatial and temporal heterogeneity of process, which supposedly it can be observed only in certain stages of tumor progression and only in some tumors [10, 11]. These inconveniences may also explain the relative discrepancy existing between the promising obtained results in different studies on quantification of potential therapeutic targets and poor results in clinical trials [35]. However, the potential of cadherins study within EMT process is related to the plasticity of the epithelia, meaning that some studies pointed that blocking the E-cadherin repression pathways can restore its expression and intercellular connections, as well as the response of cancer cells to targeted therapies [36–39]. Even in the case of urothelial carcinoma, there

are some evidences that the reduction in the expression of transcription factors leads to the restoration of E-cadherin expression and the response to conventional therapy [39]. Also, blocking aberrant expression of N-cadherin is associated in various cancers with reduced resistance to therapy and the improvement of prognosis [40–42].

These evidences are arguments to characterize more precisely the expression of E- and N-cadherins in different tumor compartments. The cadherin switch and its relationship with other mechanisms involved in deregulation of cell cycle, proliferation apoptosis and angiogenesis can be the basis of solid clinical trials based on effective biomolecular connections with impact on already identified therapeutic targets within EMT process.

## Conclusions

In this study, for high-grade, invasive and metastatic urothelial carcinomas we found an E-cadherin decreased expression and increased N-cadherin expression in both intratumoral and advancing edge compartments. The cadherin switch presence, measured as the immunostain variation for the two proteins is significantly higher into the advancing edge. The presence of N-cadherin in intratumoral compartment designates it as one actively involved in epithelial-mesenchymal transition process. The analysis of cadherin switch can be used for identification of superficial urothelial carcinomas with invasion and metastasis potential.

## Conflict of interests

The authors declare that they have no conflict of interests.

## Author contribution

Andrei Ioan Drocaș and Paul Ioan Tomescu equally contributed to the manuscript.

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