Immunooexpression of transcription factors in urothelial bladder carcinomas

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Abstract
Transcription factors play a central role in the epithelial–mesenchymal transition (EMT), which is one of the biomolecular mechanisms involved in the progression of urothelial carcinomas of the bladder (UCB). In this study, we analyzed the immunoeexpression of Twist 1, Snail, Slug and β-catenin in relation to histopathological prognostic parameters of UCB. The obtained results indicated the association of Snail and β-catenin expression with low grade and early stage of UCB, as well as the association of Twist 1 and Slug expression with high grade and advanced stage lesions. The specific or sequential action of transcription factors in the bladder tumoral EMT may be useful for identifying the aggressive lesions.

Keywords: urothelial carcinoma, Twist 1, Snail, Slug, β-catenin.

Introduction
Urothelial carcinomas represent over 90% of malignant tumors in the bladder [1]. The lesions ranked the ninth place worldwide, being responsible for about 165 000 deaths annually [2, 3].

Urothelial carcinoma of the bladder (UCB) raises particular socio-economic problems, being the most expensive malignant tumors per patient due to the recurrence, survival, need to monitor by repeated tumor transurethral resections (TURs) and sometimes by cystectomy [4]. In this regard, numerous biomolecular mechanisms involved in the initiation and progression of urothelial carcinomas have been investigated, which has led to the identification of divergent pathogenic pathways for non-invasive and invasive lesions, as well as to the initiation of clinical trials having different therapeutic targets [5, 6]. However, the current treatment protocols fail to increased the survival rate (less than 60% at five years), recurrence risk (over 50%) and quality of life which remains the lowest reported to all cancers [4, 7].

One of the biomolecular mechanisms involved in the progression of UCB is the epithelial–mesenchymal transition (EMT) [8, 9]. In this process, transcription factors occupy a central place, being involved in regulating the cadherin switch, loss of epithelial phenotype and the acquisition of the mesenchymal phenotype, overexpression of some growth factors [10–12]. The most studied transcription factors in the urinary bladder in relation with EMT are represented by Twist 1, Snail, Slug and β-catenin (Table 1).

In order to immunostaining, the sections were prepared for incubation with primary antibodies (dewaxing in xylene, rehydrating in alcohols, endogenous enzyme and unspecific blocking, microwaving for antibody retrieval) at 4°C, overnight. The working system was represented by Labeled Streptavidin–Biotin (LSAB) 2 system (Dako, Redox, Romania, code K0675), and we used 3,3'-diaminobenzidine tetrahydrochloride (Dako, Redox, Romania, code K0675), and we used 3,3'-diaminobenzidine tetrahydrochloride (Dako, Redox, Romania, code K0675).
code K3468) as chromogen. External positive controls and external negative controls were used for the immunostaining reactions.

<table>
<thead>
<tr>
<th>Table 1 – The antibodies and immunostaining protocol</th>
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<tbody>
<tr>
<td>Antibody</td>
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</tr>
<tr>
<td>Twist 1</td>
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<tr>
<td>Snail1 (Snail)</td>
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<td>Snail2 (Slug)</td>
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<td>β-catenin</td>
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</table>

EDTA: Ethylenediaminetetraacetic acid.

The assessment of reactions was done by using a score resulting by multiplying of labeled cells and intensity of reactions. Referring to intensity, the reactions were scored as 1 (mild), 2 (moderate) and 3 (strong) and depending on the number of labeled cells, the reactions were scored as 1 (<25% labeled cells), 2 (26–49% labeled cells), 3 (>50% labeled cells). The final scores were considered as 1 (<25% labeled cells), 2 (26–49% labeled cells), 3 (>50% labeled cells). The final scores were considered low for 1–4 and high for 6–9 values. We used χ² (chi)-square and Pearson tests within SPSS 10 software for the statistical analysis, for p-values less than 0.05 the results being considered significant. For the images acquisition, the Nikon Eclipse E600 microscope equipped with Lucia 5 software was used. The local ethical committee approved the study.

Results

The clinicopathological data analysis revealed in this study the predominance of UCB in male patients (80.9%), most with over 50 years (95.2%) with a mean age at diagnosis of 63.2±8.7 years. Most of the analyzed cases were high-grade carcinomas (52.3%), with the muscularis propria invasion (64.2%), without lymph node metastasis (92.8%) and in the tumor stage II (61.9%) (Table 2).

<table>
<thead>
<tr>
<th>Table 2 – Clinicopathological parameters</th>
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<tr>
<td>Clinicopathological parameters</td>
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<tr>
<td>Age [years]</td>
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<tr>
<td>Gender</td>
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<tr>
<td>Degree of differentiation</td>
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<td>Depth of invasion (T)</td>
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<tr>
<td>Lymph node status (N)</td>
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<tr>
<td>Stage</td>
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</table>

LG: Low grade; HG: High grade.

The assessment of performed immunohistochemical reactions indicated differences in the expression of the transcription factors in relation to the histopathological analyzed parameters.

Twist 1 immunoexpression

Twist 1 immunoexpression was identified at the nuclear level in all analyzed cases. The reactions were present both in tumor cells and stromal elements represented by endothelial cells, fibroblasts, lymphocytes, plasma cells, some macrophages.

Twist 1 immunoreactions were superior in the case of high-grade carcinomas with deep invasion and advanced stages. Thus, for high-grade carcinomas, the number of marked tumor cells was 76.6±11.8, the intensity of the reactions being moderate or strong, and the mean final score being 8.3. By comparison, for low-grade carcinomas the number of labeled cells was 56.7±11.2, moderate or increased intensity and the final score 6, difference which was statistically significant (p=0.000, chi-square test) (Figure 1). The analysis of Twist 1 reactions in relation to the invasion depth indicated higher values for deep invasion carcinomas compared to invasive lesions in the lamina propria, but the aspects were statistically insignificant (p=0.580, chi-square test). Thus, the highest mean values of the final Twist 1 score were observed in pT2-T4 lesions, in which the number of labeled cells was comprised between 45–95% compared to pT1 lesions in which the value was 56.2±8.5, the reactions intensity being strong or moderate for both categories. The aspects were similar for the analysis of Twist 1 expression in relation to the tumor stage. Although the number of marked cells was higher for carcinomas that had lymph node metastases compared to those without metastases (90.0±5 vs. 65.4±14.3), the intensity of the reactions was strong or moderate for both categories, and the statistical differences were insignificant (p=0.348, chi-square test) (Table 3).

Snail immunoreactions

Snail immunoreactions were observed at the nuclear and cytoplasmic level in 34 cases, respectively 80.9% of the analyzed cases. Similarly to the other transcription factors analyzed, the reactions were present in stromal mononuclear elements. We found differences in Snail expression relative to histological analyzed parameters. Thereby, in the case of low-grade carcinomas (48±8.6 labeled cells, moderate/strong intensity and average score 6.2), the immunoreactions were significantly superior to those of high grade (38.2±11.8 labeled cells, variable intensity and mean score 4.0) (Figure 1). Although the reactions were superior in the case of superficial tumors (pT1) without lymph node metastases (pN0) and initial stages (tumor stages I and II), the differences were statistically insignificant (p>0.05, chi-square test) (Table 3).

| Table 3 – Transcription factors immunoexpression and their relation with histopathological parameters |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|
| Histological parameters | Twist 1 score, p* level | Snail score, p* level | Slug score, p* level | β-catenin score, p* level |
| Differentiation degree | LG | 6.0 | 6.2 | 1.8 | 6.7 |
| | HG | 8.3 | 4.0 | 4.7 | 4.8 |
| p | p=0.000 | p=0.034 | p=0.037 | p=0.036 |

*p level
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<table>
<thead>
<tr>
<th>Histological parameters</th>
<th>Twist 1 score, $p^*$ level</th>
<th>Snail score, $p^*$ level</th>
<th>Slug score, $p^*$ level</th>
<th>$\beta$-catenin score, $p^*$ level</th>
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<td><strong>Depth of invasion</strong></td>
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<td>$p=0.580$</td>
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<td><strong>Lymph node</strong></td>
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<td>N1</td>
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<td>$p=0.348$</td>
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<td><strong>Tumor stage</strong></td>
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<td>I</td>
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<td>II</td>
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<td>III</td>
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<tr>
<td>IV</td>
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<td>$p=0.498$</td>
<td>$p=0.107$</td>
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$p^*$ level (chi-square test); LG: Low grade; HG: High grade.

**Figure 1** – Urothelial carcinoma of the bladder: (A) Low grade, Twist 1 immunostaining (×100); (B) High grade, Twist 1 immunostaining (×100); (C) Cases distribution depending on tumor grading and Twist 1 scores; (D) Low grade, Snail immunostaining (×100); (E) High grade, Snail immunostaining (×100); (F) Cases distribution depending on tumor grading and Snail scores.

**Slug immunoexpression**

Slug immunoexpression was found in 30 cases, which represented 71.4%, the immunostaining being observed in nuclear and cytoplasmic level, both in tumor cells, as well as in the stromal elements.

The reactions were higher in high-grade carcinomas and in advanced stages. In the case of low-grade tumors, the number of labeled cells was $22.7\pm8.2$, low or moderate intensity of reactions and an average score of 1.8, while for high-grade tumors the number of positive cells was $41.3\pm9.9$, variable reactions intensity and mean Slug score of 4.7, which were statistically significant ($p=0.037$, chi-square test) (Figure 2).

In relation to the depth of invasion, the number of labeled cells was $24.1\pm5$ in pT1 lesions, $33.2\pm11.9$ in pT2, $50\pm5$ for pT3 and $50\pm8.6$ for pT4 cases. The intensity of the Slug reactions was low or moderate in pT1/T2 carcinomas, and strong or moderate in pT3/T4 lesions, the mean Slug scores being 2.4 in the case of pT1, 3.0 in pT2, 6.3 in pT3 and 8.0 in pT4 cases, aspects that were statistically significant ($p<0.000$, chi-square test). The aspects were similar in the case of the analysis of the reactions in relation to the tumor stage, the mean values of the Slug score being significantly higher in the advanced stages compared to the stages I and II ($p=0.000$, chi-square test). Although the number of marked cells was higher for metastatic carcinomas compared to those without metastases, respectively $48.3\pm7.6$ and $32.9\pm12.5$, the intensity of the reactions was variable and the statistical differences of the mean Slug scores (6.7 in pN1 and 3.5 in pN0 cases) were insignificant ($p=0.148$, chi-square test).

In this study, we did not find any differences in the localization of the Snail and Slug expression in relation to the histopathological parameters.
**β-catenin immunoreaction**

β-catenin immunoreaction was identified predominantly at the membranous and cytoplasmic level as well as in rare nuclei of tumor cells in 32 cases, which represented 76.1% of the investigated group. Also, the immunostaining was observed in peritumoral stromal elements. In this study, we found significantly higher levels of β-catenin expression in low-grade carcinomas (62.3±13 labeled cells, moderate/strong intensity and average score 6.7) compared to high-grade lesions (47±12 marked cells, moderate/strong intensity and average score 4.8) (Figure 2).

We have found the predominance of membrane β-catenin immunostaining in the case of low-grade carcinomas with superficial invasion (pT1) and cytoplasmic and nuclear in the case of high-grade carcinomas and invasion at least in muscularis propria (pT2-T4).

Also, the reactions were superior in case of lamina propria (pT1) invasive carcinomas, with no lymph node metastases (pN0) and in the initial stages (stages I and II), but the aspects were without statistical significance (p>0.05, chi-square test) (Table 3).

The analysis of the percentage values of the investigated markers indicated significant positive linear correlations between Twist 1 and Slug (p=0.000, Pearson’s test), and between Snail and β-catenin (p=0.001, Pearson’s test) (Figure 3).

Between the two groups, we found statistically insignificant negative linear relations (p>0.05, Pearson’s test). In this study, we did not find other statistical associations of marker-analyzed immunoexpression with the age and gender of patients diagnosed with UCB.

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**Discussion**

Transcription factors are part of the largest family of proteins that are involved in the transmission of genetic information from DNA sequences to messenger RNA using RNA polymerase [16, 17]. The transcription is a complex process in which there may be several factors that co-operate in order to regulate a genetic sequence, factors that in turn can have numerous regulating mechanisms for their expression [18, 19]. Targeting the transcription factors is an effective way of controlling the gene expression, an aspect that is an attractive target for anticancer therapy [14, 20, 21].

Transcription factors are involved in regulating the carcinomas EMT process, which is a complex biomolecular mechanism that plays a role in the progression of lesions, respectively in invasion and metastasis [8–10]. The process consists in acquiring a mesenchymal migratory
phenotype of cancer cells, and altering the epithelial phenotype and intercellular adhesion system, whose the main component is E-cadherin [10, 11]. At the same time, the process can be induced by numerous other molecular pathways, like as Wnt, TGF-β, Hedgehog, Notch, whose interconnection with tumor EMT has been demonstrated [11]. The studies for transcription factors in EMT bladder tumors are relatively few, and the process seems to be temporary and only in some tumor compartments [8]. This aspect, along with the large number of proteins involved in EMT and interaction with other molecular pathways of tumor progression, advocates the investigation of transcription factors as initiators and process regulators. In our study, we analyzed the immunoexpression in UCB of some factors involved in the tumor EMT, represented by Twist 1, Snail and Slug.

Twist 1 is a protein that is part of the basic helix-loop-helix (bHLH) family and is involved in EMT by loss of epithelial profile expression and intercellular adhesion molecules, respectively of E-cadherin [8, 22]. It also regulates overexpression of N-cadherin and promotes the tumor motility and invasiveness [8, 22]. Twist also plays a role in promoting tumor angiogenesis and generating cancerous stem cells [8, 22, 23]. Twist 1 was analyzed in EMT of carcinomas with different locations such as colorectal, breast, prostate, pulmonary, hepatic, oral cavity, ovarian or esophageal, the high expression being associated with aggressive lesions [24–27].

In our study, the Twist 1 immunoreactions were present in all the analyzed cases at nuclear levels as well as in the stromal elements. There are studies that have identified the Twist expression in cytoplasm, with reactions seen in about 40% of the tumors analyzed [23, 28]. In our study, the Twist immunoreactions were present in all the analyzed cases at nuclear levels as well as in the stromal elements. There are studies that have identified the Twist expression in cytoplasm, with reactions seen in about 40% of the tumors analyzed [23, 28].

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diminished β-catenin membrane expression is indicated for high-grade and aggressive carcinomas [51].

In the literature, there have been studies that have shown a positive association of cytoplasmic and nuclear expression of transcription factors and the presence of activated stromal fibroblasts, association that appears to be involved in the invasive phenotype of UCB [37]. In this study, for all the analyzed factors, we found the presence of positive stromal elements associated with tumor cells, both in the fibroblasts, as well as in the inflammatory mononuclear and endothelial cells. The appearance may suggest the interrelation of the analyzed transcription factors with tumor micromedium and tumor paracrine mechanisms.

There are data in the literature that indicate different relations between transcription factors of EMT. Thus, Snail and Slug factors have synergistic effects, but experimental animal models indicate different roles at different stages of tumor progression [36]. Also, synergistic and even complex formation between β-catenin and Snail for Wnt pathway activation is indicated [52].

In our study, we found positive linear correlations of β-catenin–Snail and Slug–Twist expression, and negative linear relations between these two groups of factors, which supports the sequential involvement in urothelial carcinogenesis or their specific expression for different histological subtypes of UCB.

Conclusions

In this study, Snail and β-catenin immunoexpression was associated with low-grade and early stage UCB, while Slug and Twist 1 immunoexpression was associated with high-grade and advanced lesions. The obtained results support the specific or sequential action of the transcription factors in the bladder EMT. The markers analyzed in this study may be useful for identifying UCB with progression potential and for better stratification of patients for therapy.

Conflict of interests

The authors declare that they have no conflict of interests.

References

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