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Original article

Expression profile of epithelial-mesenchymal transition markers in non–muscle-invasive urothelial carcinoma of the bladder: Correlation with intravesical recurrence following transurethral resection

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Abstract

Objectives: To evaluate the expression of molecular markers involved in epithelial-mesenchymal transition (EMT), a key process mediating the progression of malignant tumors, in non–muscle-invasive urothelial carcinoma of the bladder (NMIUCB) to clarify the significance of these markers as predictors of intravesical recurrence in patients treated with transurethral resection (TUR).

Materials and methods: Expression levels of 13 EMT markers, including E-cadherin, N-cadherin, β-catenin, γ-catenin, fibronectin, matrix metalloproteinase (MMP)-2, MMP-9, Slug, Snail, TWIST, vimentin, ZEB1, and ZEB2, in TUR specimens obtained from 161 consecutive patients with NMIUCB were measured by immunohistochemical staining.

Results: Of these 13 markers, significant differences in the incidence of intravesical recurrence were noted according to expression levels of E-cadherin, N-cadherin, MMP-2, MMP-9, and TWIST. Univariate analysis also identified expression levels of E-cadherin, N-cadherin, MMP-2, MMP-9 and TWIST, in addition to the tumor size, pathological T category, and concomitant carcinoma in situ, as significant predictors of intravesical recurrence-free survival. Of these significant factors, expression levels of E-cadherin, MMP-9, and TWIST; tumor size; and concomitant carcinoma in situ appeared to be independently associated with intravesical recurrence-free survival on multivariate analysis. Furthermore, there were significant differences in recurrence-free survival according to positive numbers of these 5 independent risk factors (i.e., positive for 0 or 1 factor vs. positive for 2 factors vs. positive for 3 or more factors).

Conclusions: Consideration of expression levels of EMT-associated markers in TUR specimens, in addition to conventional prognostic parameters, would contribute to the accurate prediction of intravesical recurrence following TUR for NMIUCB.

Keywords: Epithelial-mesenchymal transition; Non–muscle-invasive urothelial carcinoma of the bladder; Intravesical recurrence

1. Introduction

More than 80% of newly diagnosed bladder cancers are classified as non–muscle-invasive tumors that are confined to the urothelium or infiltrate the lamina propria. The initial standard management for patients with non–muscle-invasive urothelial carcinoma of the bladder (NMIUCB) is complete transurethral resection (TUR) of the visible tumor burden. However, postoperative intravesical recurrence has been reported to occur in 30% to 80% of patients undergoing TUR for NMIUCB [1]. Accordingly, it is necessary to develop systems that can accurately predict the probability of intravesical recurrence following TUR to help plan appropriate postoperative adjuvant therapy and follow-up schedules for individual patients with NMIUCB.

Although intensive studies have been conducted to identify risk factors closely associated with intravesical recurrence of NMIUCB, such as the tumor size, stage, grade, multiplicity, and microvascular invasion, consistent findings regarding this issue have not been obtained [1–3]. Furthermore, NMIUCB has been shown to be characterized by unique biological features reflecting heterogeneous genetic backgrounds [4], suggesting the limitations of

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predicting postoperative outcomes in patients with NMIUCB using conventional clinicopathological parameters alone. Accordingly, several investigators have evaluated the significance of various types of molecular marker as predictors of intravesical recurrence following TUR [5–11]. For example, the utility of the urine fibroblast growth factor receptor (FGFR)-3 mutation assay was reported; that is, FGFR-3 mutations were detected in 78% of postoperative urine samples from recurrent cases with FGFR-3 mutations in the tumor, while no mutations were detected in the urine of 15 nonrecurrent cases [8]. To date, however, no molecular markers have been widely introduced into routine clinical practice for predicting outcomes in patients with NMIUCB who are treated with TUR.

Epithelial-mesenchymal transition (EMT) is a multistep process in which polarized epithelial cells are converted into motile mesenchymal cells, accompanying alterations in various cellular phenotypes associated with adhesion, morphology, architecture, and migration [12]. In the field of cancer research, EMT has also been demonstrated to be involved in the progression of a wide variety of malignant tumors [13]. Furthermore, recent studies have shown the importance of multiple complex signaling pathways in the induction of EMT in tumor cells; therefore, numerous molecular markers mediating the execution of EMT were identified, and the utility of these markers as prognostic indicators in some types of malignant tumor has been reported [14–16]. In bladder cancer, there have been several studies showing the significance of EMT markers in the progression of MIUCB [17–19]; whereas it remains largely unknown whether these markers have an effect on the prediction of postoperative recurrence in patients with NMIUCB.

Considering these findings, we assessed the expression patterns of multiple EMT markers, including E-cadherin, N-cadherin, β-catenin, γ-catenin, fibronectin, matrix metalloproteinase (MMP)-2, MMP-9, Slug, Snail, TWIST, vimentin, ZEB1, and ZEB2, in TUR specimens from 161 consecutive patients with NMIUCB to investigate the association between the expression of these markers and the probability of intravesical recurrence in this patient category.

2. Patients and methods

Of consecutive patients who were treated with TUR of newly diagnosed primary bladder cancer at our institution, a total of 161 patients were subsequently diagnosed with NMIUCB (i.e., Ta- or T1-category tumor) were included in this study. The study design was approved by the Research Ethics Committee of our institution, and informed consent for conducting this study was obtained from each patient. The growth pattern of tumors was macroscopically classified into either papillary or nonpapillary type, and the tumor size was defined as the largest tumor measured with a resection loop corresponding to 1 cm long. In all of the included patients, complete resection of all visible tumors could be accomplished, and several deep muscular samples were further obtained. Irrespective of the preoperative findings, including those of urinary cytology, random bladder biopsies were performed before TUR for all patients. Histopathological examinations were carried out by a single pathologist according to the 2010 American Joint Committee on Cancer TNM classification system.

In this series, the indication for adjuvant intravesical instillation therapy was generally determined according to the pathological findings showing the presence of concomitant carcinoma in situ (CIS) or T1G3 disease or both; therefore, bacillus Calmette-Guérin was administered for most patients who received adjuvant intravesical instillation therapy. Follow-up after TUR of NMIUCB was performed based on the schedule, as previously described [10]: cystoscopy and urinary cytological examination were performed every 3 to 6 months for 3 years after TUR, and then every 6 to 12 months until 5 years after TUR, and intravenous pyelography was performed every 6 to 12 months for 5 years after TUR. When detecting tumors or hyperemic mucosa by cystoscopy or positive findings on urinary cytology, TUR of the tumor or transurethral biopsy of the abnormal region or a combination of both was performed.

Immunohistochemical staining of TUR specimens was performed as previously described [10]. Briefly, sections from paraffin-embedded tissues were deparaffinized by xylene and rehydrated in decreasing concentrations of ethanol. After blocking endogenous peroxidase, sections were boiled in 0.01 M citrate buffer for 10 minutes and incubated with 5% normal blocking serum in Tris-buffered saline for 20 minutes. The sections were then incubated with the following antihuman antibodies for 60 minutes: E-cadherin mouse monoclonal antibody (NCH-38, Dako, Carpinteria, CA); N-cadherin mouse monoclonal antibody (6G11, Dako); β-catenin mouse monoclonal antibody (E-5, Santa Cruz Biotechnology, Santa Cruz, CA); γ-catenin mouse monoclonal antibody (H-1, Santa Cruz Biotechnology); fibronectin mouse monoclonal antibody (EP5, Santa Cruz Biotechnology); MMP-2 mouse monoclonal antibody (42-5D11, Daiichi Fine Chemical, Toyama, Japan); MMP-9 mouse monoclonal antibody (56-2A4, Daiichi Fine Chemical); Slug mouse monoclonal antibody (A-7, Santa Cruz Biotechnology); Snail mouse monoclonal antibody (G-7, Santa Cruz Biotechnology); TWIST mouse monoclonal antibody (Twist2Cia, Santa Cruz Biotechnology), vimentin mouse monoclonal antibody (V9, Dako); ZEB1 rabbit polyclonal antibody (H-102, Santa Cruz Biotechnology); and ZEB2 mouse monoclonal antibody (E-11, Santa Cruz Biotechnology). The sections were subsequently incubated with biotinylated goat antimouse or antirabbit IgG (Vector Laboratories, Burlingame, CA) for 30 minutes. After incubation in an avidin-biotin peroxidase complex for 30 minutes, the samples were exposed to diaminobenzidine