RESEARCH ARTICLE

Prognostic Significance of Nestin Expression in pT1 High-Grade Bladder Urothelial Carcinoma Patients Treated with Intravesical BCG

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Abstract

Background: Possible roles of nestin expression in terms of predicting intravesical BCG therapy response in T1 high grade bladder cancer patients were investigated. Materials and Methods: T1 high grade bladder cancer patients who were treated with intravesical BCG between 1990-2009 were included. Immunohistochemical staining for nestin expression was performed. Nestin(+) and nestin(-) patients were compared in terms of recurrence and progression rates. Results: Sixty-three patients were included and median follow-up time was twenty-five months. After staining; 33 patients (52.4%) were classified as nestin (+) and 30 (47.6%) as (-). Nestin (+) patients were more likely to recur compared to nestin (-) patients (60.6% *vs*. 30%, p<0.05). Progression rates were also higher in nestin (+) patients, although this result did not reach statistical significance (15.2 % *vs*. 10 %, p=0.710). Conclusions: Nestin expression, which seems effective in predicting recurrence, appears to have a potential role in the urothelial carcinoma tumorigenesis. Patients with high grade bladder cancer and positive nestin expression need close follow-up and might be informed about more tendency to recur. Further comprehensive studies including larger patient cohorts may clarify the role of nestin in bladder cancer.

Keywords: Bladder cancer - BCG treatment - response - nestin expression

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Introduction

Bladder cancer is the fourth most frequent neoplasia in men and ninth in women (Jemal et al., 2009). More than 90% of bladder cancer is urothelial carcinoma and 70-80% of patients have non-muscle-invasive tumors at presentation (Witjes and Hendricksen., 2008). Biological behaviour of non-muscle-invasive bladder cancer (NMIBC) cancer is not known exactly for today and many studies have been done for predicting prognosis of NMIBC.

T1 tumors are usually high grade and represent a heterogenous population in terms of prognosis, management and follow-up options. Almost 80% of T1 patients will have a tumor recurrence if treated only by transurethral resection (Heney, 2005). Transurethral resection of bladder tumor (TURB) and then intravesical instillation of bacillus Calmette-Guérin (BCG) is suggested as the first line treatment for this disease (Manoharan and Soloway., 2005; Okamura et al., 2010). Recurrence rate is 16% to 40% and progression rate is 4.4% to 40% after BCG treatment (Malkowicz et al., 1990; Cookson and Sarosdy., 1992; Jimenz-Cruz et al., 1997; Hurle et al., 1999; Okamura et al., 2012). Early cystectomy is a good option but obviously overtreats potential responders to BCG immunotherapy. Therefore, we would like to know whether patients will have a good response to BCG treatment or not and it is important to identify reliable prognostic factors for response to BCG treatment. Although some tumor markers such as p53 and retinoblastoma protein (pRb) have been studied for this purpose previously, they could not take place in routine clinical practice (Lima et al., 2012).

Nestin is a class VI intermediate filament protein expressed in stem/progenitor cells during the development of the central nervous system (Toshiyuki et al., 2011). Nestin is detected in various types of tumors and is involved in malignant processes (Takano et al., 1996; Yang et al., 2000; Almazan et al., 2001; Lardon et al., 2002; Toshiyuki et al., 2011). Proliferating vascular endothelial cells in tumor tissues also highly express nestin and nestin is therefore considered to be closely related with tumor angiogenesis (Gravdal et al., 2009). Ozer et al reported in a previous study that T1 high grade bladder cancers possess higher angiogenetic activity resulting in a greater rate of progression or recurrence rates (Ozer et al., 1999). We hypothesized that nestin expression might have a role in predicting BCG response because of its association with

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tumor angiogenesis. Hence, we explore the effect of nestin expression for predicting BCG response in high grade T1 bladder cancers in the present study.

Materials and Methods

Patient population

Medical records of patients listed in our cancer database with diagnosis of bladder cancer between the years of 1990-2009 were retrospectively reviewed. The study protocol was approved by the institutional review board. Patients with primary T1 high grade urothelial carcinoma ±carcinoma in situ who were treated with intravesical induction BCG and older than 18 years old were enrolled. Patients with a history of pelvic radiotherapy, prior intravesical chemotherapy and previous diagnosis of upper urinary tract urothelial carcinoma were excluded. All patients underwent white light cystoscopy and complete TURB including muscle in the specimen and resected tissues were examined by two experienced uropathologists (BT and KY). Second resection was not performed. After TURB, all patients received intravesical induction BCG for 6 weeks without maintenance. One vial of BCG was diluted and mixed with 50 cc of saline, intravesical instillation was administered by 10 Fr nelaton urethral catheter. Urinalysis and urine cultures were performed before all instillations and therapy was postponed in any doubt of active urinary tract infection. Follow-up consisted of white light cystoscopy every 3 months for the first 2 years, every 6 months between second and fifth years, and then annually. Recurrence was considered as histologic evidence of malignancies of the same or lower stage and grade. Tumor progression was defined as histologically confirmed muscle-invasive bladder cancer or evidence of metastatic disease.

Pathology

TURB specimens were fixed in 10%formalin and embedded in paraffin. All cases were re-evaluated by two uropathologists (BT and KY). Tumors were staged according to the TNM staging system and graded according to the grading scheme proposed by World Health Organization (WHO) (Cheng et al., 2009; Epstein et al., 1998).

Immunohistochemistry

Sections from the paraffin blocks of the most representative tumor tissues from the included patients were taken on to poly-L-lysin coated slides. Standard streptavidin biotin immunperoxidase method was performed for immunostaining with Nestin antibody (Bioss, bs-0006R, 1:100 dilution, USA). The sections were deparaffinized, rehydrated and endogenous peroxidase activity was blocked using a 0.3% solution of hydrogen peroxide at room temperature for 10 minutes. The sections were then heated in citrate buffer (0.01mol/L, pH 6) for epitope retrieval in a microwave oven (3 times for ten minutes at 700W), then allowed to cool to room temperature for 20 minutes. Primary antibody was applied for 30 minutes at room temperature and washed in TRIS buffer. Linking antibody and streptavidin peroxidase complex (Invitrogen, Histostain Plus, 85-8943, USA) were added consecutively for ten minutes at room temperature and washed in TRIS buffer. Peroxidase activity was visualised with 3,3'-diaminobenzidinetetrachlorur (DAB) (Sigma Chemical Co, St. Louis, Missouri, U.S.A), applied for 5 minutes. The sections were counterstained with Mayer's hematoxyline. Astrocytoma section as positive control was also stained simultaneously. Negative control was stained by omitting the primary antibody incubation. All of the tumor tissue in the section was selected for immunohistochemical evaluation. Cytoplasmic expression of tumor cells was scored. The expression was scored semiquantitatively as 0: no cells stained, 1: between 0 and 10% stained positive, 2: between 10 and 50% stained positive, 3: if more than 50% stained positive. The percentage of nestin positive staining area was established by calculating the average value of positive staining area to the total area on a minimum of 10 high power fields. In our study the staining pattern of 3 was evaluated as nestin positive (Figure 1A); the staining pattern of 0.1 and 2 were evaluated as nestin negative (Figure 1B) (Enache et al., 2012). These two groups were compared in terms of recurrence and progression.

Statistical analysis

The statistical software package SPSS 20.0 version (SPSS Inc, Chicago, IL, USA) was employed for data management and analysis. Data were summarized with percentage, distribution, mean, standart deviation and median values. Chi-square test and Fisher's exact test were performed. Significance was set at $p \leq 0.05$.

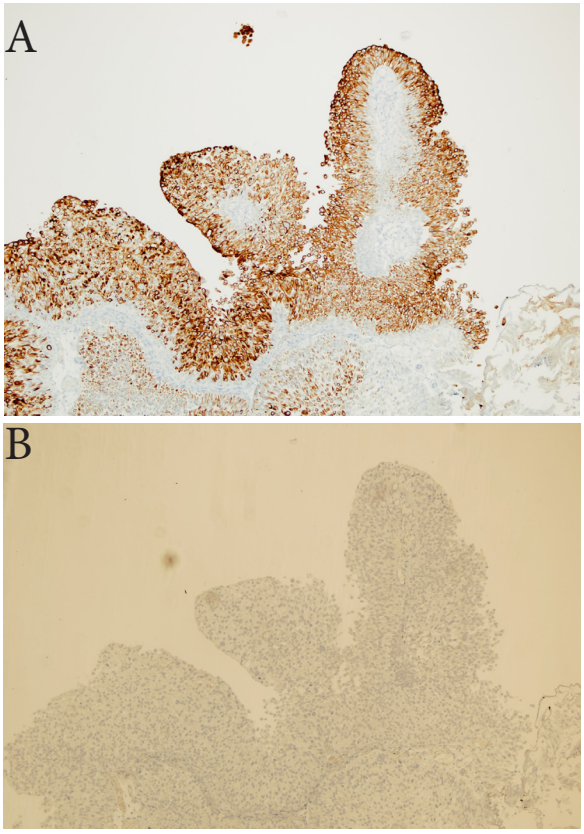

Figure 1. (A) Nestin (+) staining pattern. More than 50 % of cells were stained with Nestin antibody. (B) Negative control for nestin staining

Results

Among the sixty-three patients eligible for study protocol, there were fifty-two (82.5%) men and eleven women (17.5%). Mean age was 67±9.4 years old. The overall median duration of follow-up was 25 (3-191) months. Thirty-seven patients (58,7%) were over 65 years old. Twenty eight (44.5%) patients had a single tumor and 35 patients (55.5%) had multiple tumors. Nineteen patients (30.2%) had concomitant carcinoma in situ (CIS) and 27 patients (42.9%) had a tumor larger than 3 cm. Twenty-nine (46%) patients had recurrence and eight (12.6%) patients had progression on follow-up. Median time to recurrence and progression was 7 (2-41) and 4 (3-33) months, respectively. Three patients underwent radical cystectomy and two patients was offered concomitant radio-chemotherapy due to progressive disease course. One patient was given three course of gemcitabin+cisplatin chemotherapy because of metastatic disease on follow-up. Two patients were lost to follow-up after diagnosis of progression. Two out of sixty-three patients who had disease progression died of disease during follow-up (3.1%).

Nestin staining was positive (+) in 33 (52.4%) and negative (-) in 30 patients (47.6%). Recurrence rates were significantly higher in nestin (+) group compared to nestin (-) group (60.6% *vs*. 30%, p=0.014) whereas other possible predictive parameters for recurrence were similar between groups (Table 1). Progression rates did not differ significantly for both nestin (+) and nestin (-) groups (15.2% *vs*. 10%, p=0.710) (Table 2).

Table 1. The association of Nestin Expression and Various Clinical Parameters with Disease Recurrence in pT1 High Grade Bladder Cancer Patients

	Recurrence (-)	Recurrence (+)	Total	p value
CIS (+)	10	9	19	
(%)	-52.6	-47.4	-100	
CIS (-)	24	20	44	0.889
(%)	-54.5	-45.5	-100	
Tumor <3 cm	19	17	36	
(%)	-52.8	-47.2	-100	
				0.827
Tumor ≥3 cm	15	12	27	
(%)	-55.6	-44.4	-100	
Solitary	18	10	28	
(%)	-64.3	-35.7	-100	
Multiple	16	19	35	0.142
(%)	-45.7	-54.3	-100	
Male	29	23	52	
(%)	-55.8	-44.2	-100	
Female	5	6	11	0.533
(%)	-45.5	-54.5	-100	
Age <65	13	13	26	
(%)	-50	-50	-100	
				0.596
Age >65	21	16	37	
(%)	-56.8	-43.2	-100	
Nestin (+)	13	20	33	
(%)	-39.4	-60.6	-100	
Nestin (–)	21	9	30	0.014
(%)	-70	-30	-100	

Table 2. The Association of Nestin Expression and Various Clinical Parameters with Disease Progression in pT1 High Grade Bladder Cancer Patients

	Recurrence (-)	Recurrence (+)	Total	p value
C=CIS (+)	18	1	19	
(%)	-94.7	-5.3	-100	
CIS (-)	37	7	44	0.417
(%)	-84.1	-15.9	-100	
Tumor <3cm	33	3	36	
(%)	-91.7	-8.3	-100	
Tumor ≥3cm	22	5	27	0.272
(%)	-81.5	-18.5	-100	
Solitary	25	3	28	
(%)	-89.3	-10.7	-100	
Multiple	30	5	35	0.723
(%)	-85.7	-14.3	-100	
Male	46	6	52	
(%)	-88.5	-11.5	-100	
Female	9	2	11	0.62
(%)	-81.8	-18.2	-100	
Age <65 years	24	2	26	
(%)	-92.3	-7.7	-100	
Age >65 years	31	6	37	0.452
(%)	-83.8	-16.2	-100	
Nestin (+)	28	5	33	
(%)	-84.8	-15.2	-100	
Nestin (-)	27	3	30	0.71
(%)	-90	-10	-100	

Discussion

Nestin is a class VI intermediate filament protein that was originally described as a neuronal stem cell marker in 1990. Physiologically, high levels of nestin expression has been described in oligodendroglial cells, ependymocytes, Sertoli cells, hair follicle cells, renal glomerular podocytes, pericytes, odontoblasts and optic nerve (Takano et al., 1996; Yang et al., 2000; Almazan et al., 2001; Lardon et al., 2002; Amoh et al., 2004). Increased nestin expression has also been reported in various tumor cells, especially in central nervous system and gastrointestinal tumors as a poor prognostic factor (Eaker and Sallustio., 1994; Toshiyuki et al., 2011). High expression levels in tumor blood vessels and proliferating endothelial cells in certain tumor types suggest a relationship between tumor angiogenesis and nestin (Kim et al., 2002; Teranishi et al., 2007; Gravdal et al., 2009; Eaton et al., 2010). From urological point of view, reduction of nestin expression has been shown to be correlated with decreased invasion and migration capability of prostate cancer cells (Gravdal et al., 2009). Furthermore, it has been detected as a stem cell marker in 100% of bladder cancer cell cultures which may suggest a possible role in bladder cancer pathogenesis (Bentivegna et al., 2010). There is also one case reported with osteoclast-rich undifferentiated carcinoma of the bladder and nestin expression (Kawano et al., 2011). Recently nestin expression was reported as one of the novel prognostic indicator of poor survival for patients with bladder urothelial carcinoma after cystectomy (Tabata et al., 2014). To our knowledge, nestin expression as a prognostic factor in non-muscle invasive urothelial carcinoma of the urinary bladder who were offered

intravesical immunotherapy or chemotherapy has not yet been evaluated. We deemed T1 high grade bladder cancer cases suitable for this purpose because of the fact that these cases comprise a heterogenous population in terms of biological behaviour. Some of these cases respond well to intravesical BCG treatment, on the other hand some of them display an aggressive disease course requiring aggressive treatments such as radical cystectomy. So it is important to predict whether patients will respond to intravesical BCG or not. Although some clinical parameters such as tumor stage and grade, coexisting CIS, tumor size and tumor number which are known as clinical prognostic factors help for prediction, there is a lack of reliable molecular prognostic factors at initial diagnosis despite intense previous research on this topic. Palou et al. recently conducted a molecular trial evaluating the role of ezrin protein expression patterns as a possible prognostic factor in T1 high grade bladder cancer cases and concluded that ezrin membrane expression <20% was significantly associated with shorter disease specific overall survival and increased progression rate. Patients in their study population were the same as our patient cohort who are T1 high grade bladder cancer treated with induction BCG only without maintenance (Palou et al., 2008). Some tumor markers such as p53, Ki-67, cyclooxygenase-2 (COX-2) and pRb gene expressions have been studied for nonmuscle invasive bladder cancer prognosis previously (Chen et al., 2012; Xu et al., 2012; Lima et al., 2012; Tabriz et al., 2013; Ghafouri-Fard et al., 2014). Analysis demonstrated that no established marker to date worked well for predicting recurrence or progression on its own, they concluded that biomarker arrays combining various markers might help in prediction for recurrence and progression in nonmuscle invasive bladder cancer. There are also studies demonstrating the association between bladder cancer prognosis and microvessel density which is a marker for tumor angiogenesis. Higher angiogenetic activity has been associated with occult lymph node metastasis in invasive bladder cancer (Jaeger et al., 1995). Ajili et al evaluated the role of microvessel density as a marker for angiogenesis and found that higher microvessel density was associated with higher risk for recurrence in T1 high grade bladder cancer (Ajili et al., 2012). Our results also agree with these findings; high levels of nestin expression as a marker of higher angiogenetic acitivity was found to correlate with higher recurrences in our study population which may reflect BCG failure. Nestin expression, which seems effective in predicting recurrence, appears to have a potential role in the early stages of urothelial carcinoma tumorigenesis. On the other hand, nestin expression does not seem to predict progression which is the late stage of tumorigenesis in this patient cohort. Development of progression in these patients may be associated with angiogenesis related factors other than nestin.

According to our results there was no significant association between tumor recurrence or progression and concomitant CIS, tumor size and number of tumors all of which are globally accepted clinical risk factors for recurrence and progression. This is not much surprising considering the fact that our cohort consist of T1 high grade bladder cancer cases which constitute the most important risk for recurrence or progression. An important limitation of the present study is that only patients treated with induction BCG without second resection or maintenance BCG were included. Our findings may be controversial in the second resection and maintenance BCG era.

In conclusion, we propose that high levels of nestin expression is associated with higher recurrence rates in patients given induction BCG for T1 high grade bladder tumors. We believe that nestin may be a promising marker for predicting BCG response in these tumors. However, further comprehensive studies including larger patient cohorts are needed.

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