

Overexpression of BDNF and TrkB in human bladder cancer specimens

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Abstract. BDNF (brain-derived neurotrophic factor) and TrkB (tropomyosin receptor kinase B) are expressed in several tumor types. However, the existence of BDNF and TrkB in human bladder cancer, especially transitional cell carcinoma (TCC), has not been established. In this study, commercial TCC tissue arrays were used. Slides of paraffin-fixed human bladder tissues included all grades of TCC (13, 30 and 22 tissue samples in grade I, II and III, respectively), superficial and invasive TCC (31 and 34 tissue samples, respectively), paired malignancy-uninvolved urothelium (35 tissue samples) and normal urothelial tissues (12 tissue samples). The intensities of BDNF and TrkB immunostaining were graded as background, mild and strong (score as 0, 1 and 2, respectively). The results showed significantly overexpressed BDNF and TrkB in TCC samples compared to normal urothelium. According to grade assignment of TCC samples, BDNF in grade III and TrkB in grade I and III appeared to be overexpressed. BDNF and TrkB were overexpressed in superficial TCC samples according to staging classification. The score between the paired TCC and its uninvolved urothelium was not statistically different. In conclusion, the existence of overexpressed BDNF and TrkB in human TCC has been demonstrated in our study. A strategy involving BDNF/TrkB blockade may be a new hope for TCC target therapy.

Introduction

Bladder cancer is the ninth most commonly diagnosed cancers in the world (1). The incidence of this disease has risen continuously since 1975 (2). More than 90% of bladder cancers are transitional cell carcinoma (TCC) pathologically,

but small number of other histological variants has also been reported, e.g., squamous cell carcinoma (SCC) and adenocarcinoma (3,4). Pathological grading of the degree of differentiation and clinical staging of the degree of invasion and metastasis are common tools to evaluate the further management and to predict the outcome of the disease. Most well and moderately differentiated TCC are low-grade and superficial while most poorly differentiated tumors are high-grade and invasive into muscle (5). Current therapies for bladder cancer include surgery, radiation, and chemotherapy depending on clinical severity, and evidence-based guidelines for bladder cancer therapies have been updated recently (6,7). However, if distant metastasis occurs, the median survival is usually less than one year despite aggressive treatment with multiple anticancer agents (8). Therefore, we need to investigate new strategies for bladder cancer treatment (9).

BDNF (brain-derived neurotrophic factor) is a member of the neurotrophin family of growth factors. BDNF exerts its effects by binding to TrkB (tropomyosin receptor kinase B) receptor, which regulates the survival and differentiation of neurons (10). Although initially recognized to be expressed in the neuronal tissues, BDNF and TrkB have also been found in a wide range of normal non-neuronal tissues of adult human. Shibayama *et al* (11) reported the existence of TrkB in the small glandular cells of small intestine and colon, α cells of pancreas, monocytes and macrophages, and epidermis, but not in human bladder. However, TrkB was found in bladder of rat (12). BDNF has also been found in normal urothelium (13).

Besides its distribution in normal tissues, BDNF and/or TrkB have also been found in tumors. BDNF was found in neuroblastoma, and TrkB was shown to mediate chemotherapy resistance (14-16). TrkB and/or BDNF were also found in other solid malignancy such as pancreatic ductal adenocarcinoma (17), prostate cancer (18), and lung cancer (19). Nevertheless, their functions in these neoplasms have not been well established. The presence of BDNF in hepatocellular carcinoma (HCC) was reported, and exogenous BDNF could induce tumor cell proliferation and up-regulate HCC cell cycle-related molecules *in vitro* (20). Multiple myeloma was also confirmed to express TrkB, and BDNF acted as a survival factor but did not trigger proliferation of these cells (21). Our previous study demonstrated the

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Key words: brain-derived neurotrophic factor, tropomyosin receptor kinase B, bladder cancer, transitional cell carcinoma, immunohistochemistry

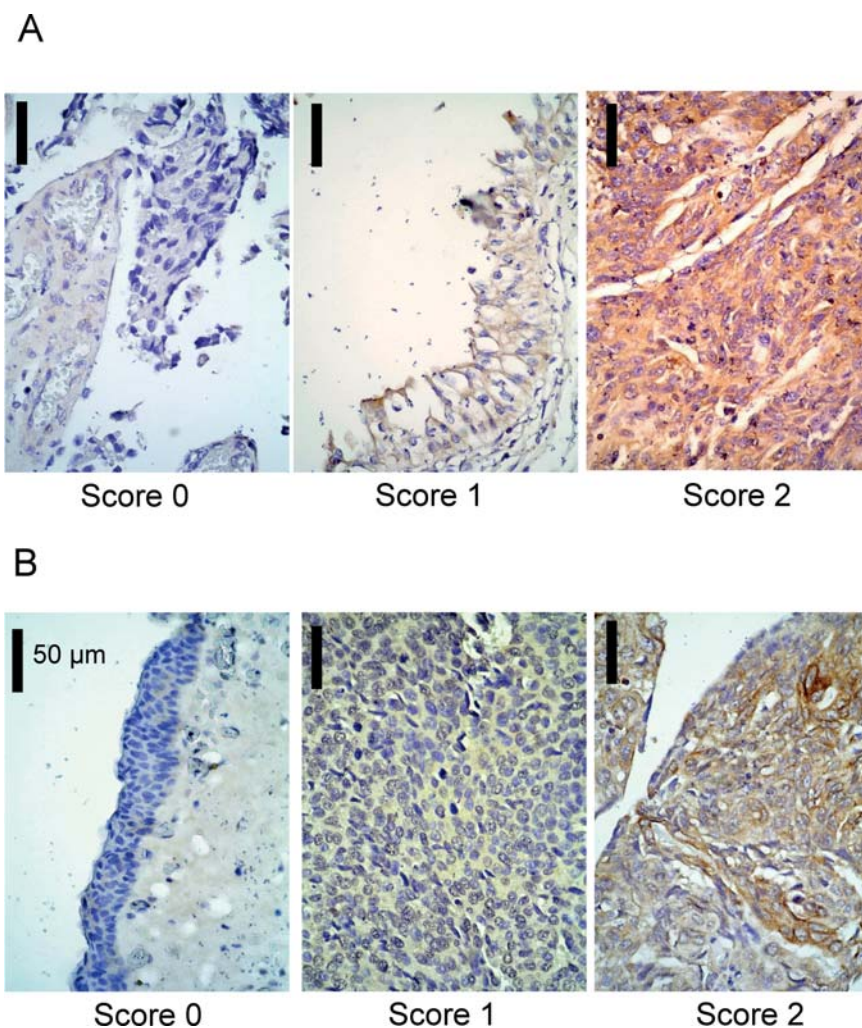


Figure 1. Immunohistochemical staining of BDNF and TrkB in TCC and normal urothelial specimens. (A) No staining (score 0), mild staining (score 1), and strong staining (score 2) of BDNF are demonstrated in three samples of grade I TCC, normal urothelium, and grade III TCC, respectively. (B) No staining (score 0), mild staining (score 1), and strong staining (score 2) of TrkB are demonstrated in three samples of normal urothelium, grade II, and grade III TCC, respectively.

existence of BDNF and TrkB in three TCC cell lines, and BDNF mediated TrkB activation is a survival signal for TCC cells *in vitro* and *in vivo* (22). Blocking TrkB elicited cytotoxicity *in vitro* and suppressed TCC xenograft growth *in vivo* (unpublished data). BDNF and TrkB played important roles in TCC progression in our preliminary study. In this study, we investigated the relationship between BDNF/TrkB expression in human specimens and their clinical grade/staging.

Materials and methods

Surgical specimens from commercial tissue arrays. Slides of paraffin-fixed human bladder tissues including all grades of TCC, matched uninvolved bladder tissues, and normal urothelial tissues were purchased from Biomax, Inc. (BL801, Rockville, MD) and Pantomics, Inc. (BLC241 and BLC 661, Richmond, CA). Excluding the absence of urothelium found on the slides, a total of 116 bladder tissues were examined for BDNF and TrkB expression. SCC and adenocarcinoma were also excluded in this study due to few samples. Examined tissues included 12 normal urothelial tissues, 35 paired non-malignancy-involved bladder tissues from TCC patients,

and 65 TCC tissues. The case number of grade I, II, and III TCC is 13, 30, and 22, respectively. According to the staging classification, the case number of superficial and invasive is 31 and 34, respectively. Some neoplastic tissues on BLC241 slide (Pantomics, Inc., certified by Dr Langxing Pan) were graded with a range (e.g., II-III), but for subsequent analysis, they were assigned to the higher grade category (i.e., III).

Immunohistochemistry (IHC). Sections were deparaffinized by non-xylene (Sigma, St. Louis, MO), and rehydrated through step-wise decrease of alcohol concentration (100, 95, 75 and 50%) for 5 min each, and then washed with PBS (phosphate-buffered saline). For antigen retrieval, the slides were then immersed in the citrate buffer at 95-100°C for 20 min. Subsequently, endogenous peroxidase was quenched with 3% hydrogen peroxide for 5 min. Blocking of background staining (protein block, Novocastra Laboratories, Newcastle upon Tyne, UK) was for 5 min, followed by rinsing with PBS. Sections were incubated overnight at 4°C with primary antibodies of anti-BDNF (1:1000, sc-546, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and anti-TrkB (1:1000, sc-8316, Santa Cruz). The following morning, horseradish

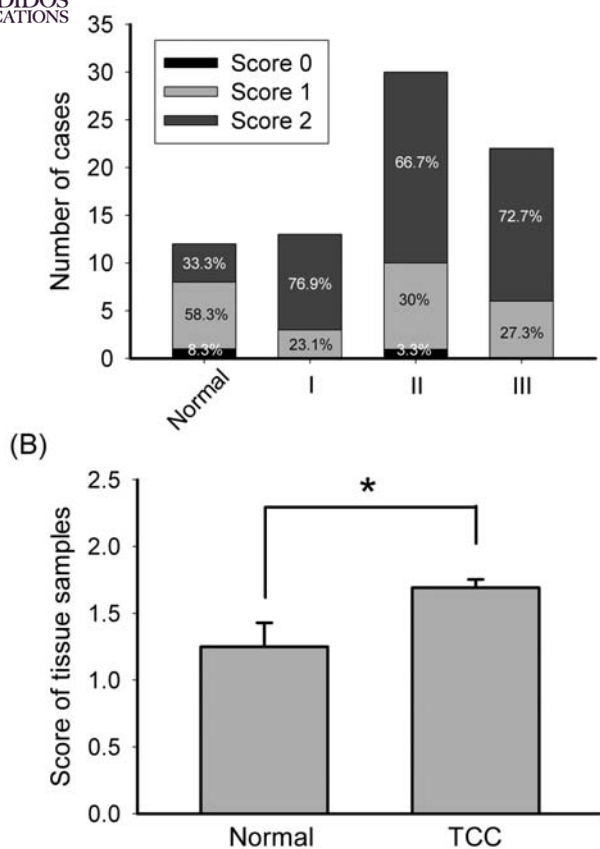


Figure 2. BDNF immunostaining score in all grades of TCC samples. (A) The distribution of BDNF score (0, 1, 2) in all grades of TCC and normal urothelial samples. (B) The difference of average BDNF score between TCC and normal urothelial tissue samples (* $p < 0.05$).

peroxidase-conjugated secondary antibodies were added for 50 min (NovoLink™ polymer, Novocastra), and substrate 3,3'-diaminobenzidine (DAB, Dako, Denmark) was added for 2-4 min. Nuclei were counterstained with hematoxylin (Dako). Primary antibody exclusion was used as a negative control. Strong, weak, and no immunoreactive staining were scored as 2, 1, and 0, respectively (23). A representative scored sample is demonstrated in Fig. 1.

Statistical analysis. Average score in each grading or staging is expressed as mean ± SEM (standard error of mean). The differences of immunoreactive score among each group and between involved and uninvolved urothelium were evaluated by nonparametric Mann-Whitney t-test and paired t-test, respectively. In all cases, significant difference was accepted at $p < 0.05$.

Results

BDNF is overexpressed in bladder cancer specimens. The distribution of BDNF score among grade I, II, III, and normal urothelium is shown in Fig. 2A. A greater percentage of strong BDNF staining (score 2) in all grades of TCC than normal urothelium was observed (33.3, 76.9, 66.7, 72.9% in normal urothelium, and grade I, II, III, respectively). The difference in BDNF score reached statistical significance

Table I. Average BDNF score of grades and stagings of TCC and normal urothelium.

| Grading/staging | Average score (mean ± SEM) | Cases |
|-------------------|----------------------------|-------|
| I | 1.77±0.12 ^a | 13 |
| II | 1.63±0.10 ^b | 30 |
| III | 1.73±0.10 ^c | 22 |
| Superficial | 1.73±0.08 ^d | 33 |
| Invasive | 1.66±0.10 ^e | 32 |
| Normal urothelium | 1.25±0.18 | 12 |

^a $p=0.052$, ^b $p=0.09$, ^c $p=0.045$, ^d $p=0.03$, and ^e $p=0.07$, respectively, compared to the score of normal urothelium.

between normal urothelium and combined TCC samples (1.25±0.18 vs. 1.69±0.06; normal urothelium vs. TCC, respectively; $p=0.03$, Fig. 2B). However, compared the score of BDNF immunostaining in each grade of TCC with that of normal urothelium, only grade III TCC showed statistically higher score (Table I). Comparing the average BDNF score between the superficial or invasive TCC and normal urothelium, only significantly higher expression of BDNF in superficial TCC was observed (Table I). The BDNF score between superficial and invasive TCC did not show significant difference ($p=0.74$).

TrkB is overexpressed in bladder cancer specimens. Immunohistochemical analysis of human specimens demonstrated that a large percentage of mild TrkB staining (score 1) was observed in normal urothelium (91.7%). Absence of strong TrkB staining (score 2) and one case of no staining (score 0) were observed in normal urothelium (Fig. 3A). In contrast, the percentage of strong TrkB staining (score 2) was increased (76.9, 46.7, and 63.6% in grade I, II, and III specimens, respectively) among TCC tissue examined (Fig. 3A). A significant difference of average TrkB score between normal urothelium and TCC samples were observed (0.92±0.08 vs. 1.44±0.09; normal urothelium vs. TCC, respectively; $p=0.0003$). Comparison of the average TrkB score, the difference between grade I and III but not II of TCC tissues and normal urothelium reached statistical significance (Table II). Furthermore, the difference of the average TrkB score reached statistical significance between superficial but not invasive TCC and normal urothelium (Table II). However, the difference of average TrkB score between superficial and invasive TCC was not statistically significant ($p=0.08$).

No difference of BDNF or TrkB immunostaining between malignancy-involved and malignancy-uninvolved urothelium. The intensities of BDNF and TrkB staining between the paired TCC and its uninvolved urothelium are shown in Fig. 4A and B. The IHC score in each grade of TCC samples was not different from that of the paired uninvolved urothelium, neither was there a difference in BDNF and TrkB score between malignancy-involved and malignancy-uninvolved urothelium in superficial and invasive TCC specimens.

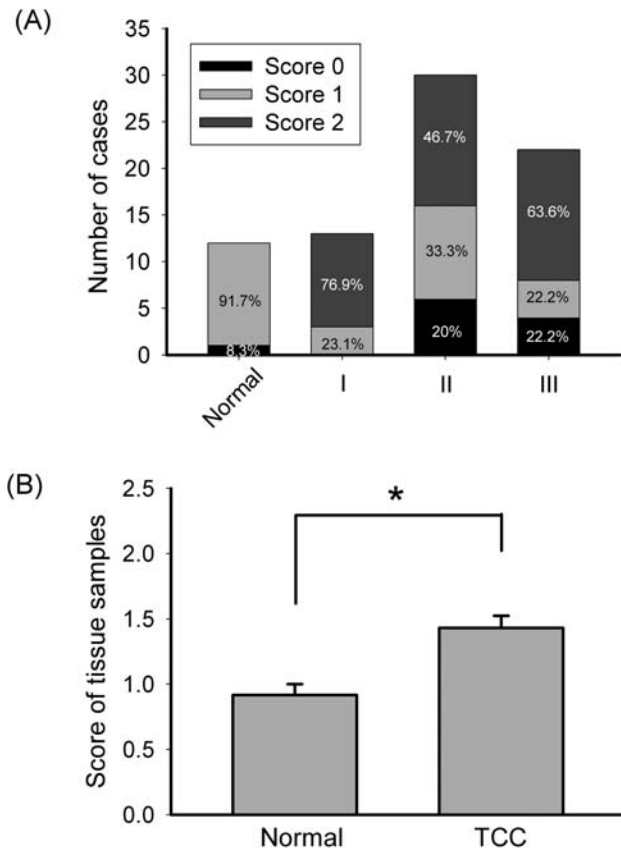


Figure 3. TrkB immunostaining score in all grades of TCC samples. (A) The distribution of TrkB score (0, 1, 2) in all grades of TCC and normal urothelial samples. (B) The difference of average TrkB score between TCC tissues and normal urothelium (* $p < 0.05$).

Table II. Average TrkB score of grades and stagings of TCC and normal urothelium.

| Grading/staging | Average score (mean ± SEM) |
|-------------------|----------------------------|
| I | 1.77±0.12 ^a |
| II | 1.27±0.14 ^b |
| III | 1.46±0.17 ^c |
| Superficial | 1.65±0.10 ^d |
| Invasive | 1.24±0.15 ^e |
| Normal urothelium | 0.92±0.08 |

^a $p = 0.0009$, ^b $p = 0.12$, ^c $p = 0.02$, ^d $p = 0.0007$, and ^e $p = 0.16$, respectively, compared to the score of normal urothelium

Discussion

The human genome contains ~90 tyrosine kinase genes (24). The products of these genes usually regulate cellular proliferation, differentiation, and motility (25). Some receptor tyrosine kinases (RTKs) which are overexpressed in cancer tissues have been characterized as oncogenes. Thus, RTK blockade is currently considered a good strategy for clinical cancer therapy (26). Overexpression of some growth factors

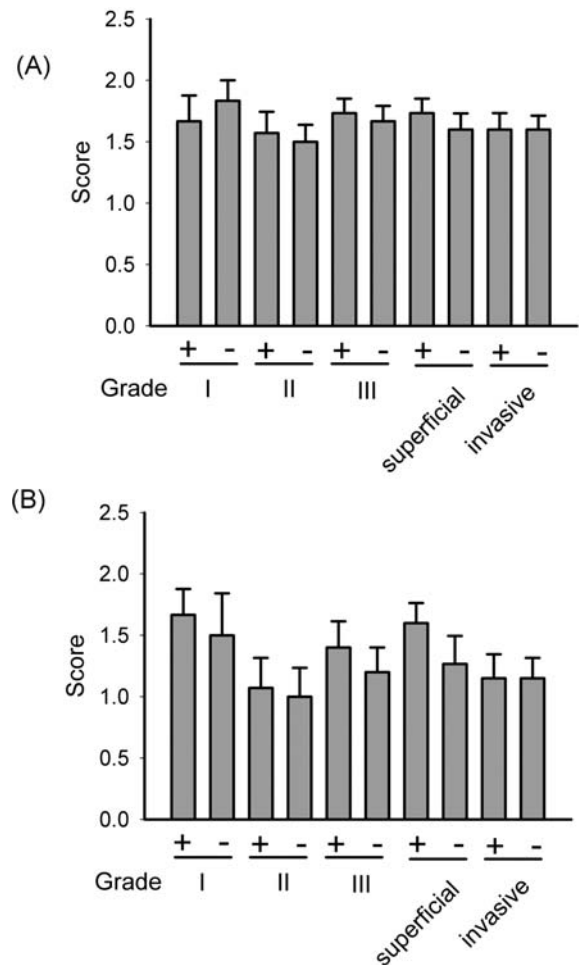


Figure 4. Staining score of BDNF and TrkB between paired TCC and uninvolved urothelium. The mean expression score between paired TCC and uninvolved urothelium (grade I, II, and III, as well as superficial and invasive TCC) was not statistically different in both immunoreactive staining of (A) BDNF and (B) TrkB.

and their specific RTKs have been found in bladder cancer, e.g., epidermal growth factor receptor (EGFR), platelet derived growth factor receptor β (PDGFR- β), fibroblast growth factor 3 (FGFR3), which are associated with poor clinical outcome (27). However, some preclinical trials of target therapies on certain RTKs showed disappointed results (28). Thus, new RTKs for bladder cancer progression should be investigated. In this study, enhanced expression of BDNF and TrkB was observed in human TCC specimens compared to normal urothelium. Thus, blockade of BDNF and/ or TrkB could become a potential strategy for TCC treatment.

Some normal urothelium also expressed mild immunostaining of TrkB in our study. It should be pointed out that no TrkB staining was found without the addition of the TrkB antibody in the IHC protocols. Therefore, the mild staining of TrkB in normal urothelium probably is not spurious, and represents the existence of low level of TrkB in normal urothelial tissue. Our results are consistent with one study (12) but differ with another (11). The reasons for such a discrepancy are not clear. However, differences in methodology, tissue sample collection and storage, and other unknown factors may contribute to the observations. For



the antibody cross-linked with unknown protein on BDNF/TrkB can not be ruled out. To definitely confirm the existence of TrkB in urothelium, other methods such as detection of the mRNA by RT-PCR (reverse transcription polymerase chain reaction) should be applied, and fresh biopsy samples without formaldehyde fixation should be used.

Although 65 human TCC tissues were examined in our study, the existence of a correlation between BDNF/TrkB expression and all pathologic grades could not be completely confirmed. Only significant BDNF overexpression in grade III TCC and TrkB overexpression in grade I and III TCC were observed. However, a trend toward enhanced expression of both, and TrkB in all grades of TCC appears to exist. It is anticipated that BDNF and TrkB would be significantly overexpressed in all grades of TCC if larger number of samples were collected and studied, but it would take years to collect the necessary tissue samples. Besides, IHC score is not a perfect method for quantification of the target protein. In future investigations, BDNF and TrkB detected by Western blotting or ELISA (enzyme-linked immunosorbent assay) in fresh TCC samples will be needed to determine the relationship between the clinical outcome and the expression levels of both proteins.

TrkB has also been demonstrated to mediate resistance to anoikis, a phenomenon of apoptosis resulting from the loss of cell-matrix interaction (28). Evidence shows that TrkB may mediate anoikis suppression by activating the PI3K-AKT pathway in ovarian cancer cells (29). Based on the literature presented above, we propose that both BDNF and TrkB may promote invasion of bladder cancer. However, only overexpressed BDNF and TrkB were observed in superficial TCC with statistical significance compared to normal urothelium. Although the levels of BDNF and TrkB expression in invasive TCC were not different from normal urothelium, they were not different from the superficial TCC either. Thus, limited number of samples might cause this discrepancy. Overexpressed BDNF and/or TrkB might be a new marker for early detection of superficial TCC according to these results. Further investigations should be conducted.

Interestingly, the expression of BDNF and TrkB was not different between paired malignant and uninvolved urothelium. The uninvolved urothelium was confirmed by optical microscopy with the diffraction-limited resolution of >200 nm. However, microscopically normal-appearing cells close to cancer lesions were found to be malignant in nature as detected by partial wave spectroscopy with a resolution of <20 nm (30). Thus, the so-called microscopically uninvolved tissue on the slide may already have malignant changes. Besides, multiple developments of urothelial carcinomas were found in many TCC patients (31). Evidence of 'field tumorigenesis' or 'clonal' expansion could explain the multifocal TCC development. For example, p53 mutant cells in cystectomy specimens could be detected in histologically normal urothelium over adjacent, remote mucosa, or preneoplastic urothelial areas (32). Thus, it means that some biomarkers or genetic changes of cancerous cells could be detected before histological transformation. Combined with the results of overexpressed BDNF/TrkB in superficial TCC, we hypothesize the possibility that overexpressed BDNF/TrkB might be a new biomarker for early

TCC detection. Collection of more TCC specimens to analyze the relationship between well-known molecular changes and BDNF/TrkB expression in histologically characterized cancer cells could give us the answer.

In conclusion, this pilot study quantified the BDNF and TrkB expression in human TCC samples. Overexpressed BDNF and TrkB were observed in commercial TCC tissue arrays. A larger number of samples among all grades and staging of TCC and normal urothelium would be needed for further investigation. Our results suggest that BDNF/TrkB blockade may be a new strategy for target therapy of TCC.

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