Original Research

Differential expression of cytokeratin 14 and 18 in bladder cancer tumorigenesis

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Impact statement

Studies have shown that expression of cytokeratins (CKs) or their altered distribution affects the bladder cancer pathogenesis and disease outcome, while the underlying mechanisms are not clear. The present study aims to explore the expression pattern of CK14 and CK18 during formation of papillary bladder cancer. The results showed that hyperplastic lesions showed significantly more CK14 and significantly less CK18 staining and invasive carcinomas showed increased CK14 immunostaining in all epithelial layers in *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN)-induced mouse model. The results indicate that altered CK14 (high) and CK18 (low) expression is perhaps an early event in bladder cancer tumorigenesis and is characteristic of both urothelial superficial pre-neoplastic and neoplastic lesions, which may provide the early diagnosis index.

Abstract

It has been previously suggested that cytokeratins (CKs) are important diagnostic and prognostic biomarkers for urothelial lesions. Hence it is imperative to understand the expression pattern of cytokeratins during formation of papillary bladder cancer, which was the objective of the current study. Expression pattern of CK14 and CK18 were examined using immunohistochemical staining in a mice model of papillary bladder cancer. Twenty female mice were divided into two groups—group 1 (NT) and group 2, which received *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) for 20 weeks plus one week without treatment. Following histological classification of bladder lesions, CK14 and CK18 immunostaining was assessed according to its distribution and intensity. In NT animals, both basal cells and umbrella cells showed sporadic positive staining for CK14 and CK18, respectively. In BBN group, hyperplastic lesions showed significantly more CK14 and significantly less CK18 staining ($P < 0.05$ in each case). Invasive carcinomas showed increased CK14 immunostaining in all epithelial layers. Cumulatively, our data indicate that altered CK14 (high) and CK18 (low) expression is perhaps an early event in bladder cancer tumorigenesis in females at least and is characteristic of both urothelial superficial pre-neoplastic and neoplastic lesions.

Keywords: Cytokeratin 14, cytokeratin 18, bladder cancer, tumorigenesis, biomarkers, lesions

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Introduction

Bladder cancer is the 17th most common malignancy in women and seventh most common malignancy in men and is one of the major reasons for cancer related mortality.[1] Even though environmental risk factors, occupational hazards, schistosomiasis, and genetic variants have been indicated as the cause of bladder cancer,[1–9] the exact etiology of bladder cancer is not known and thus presents as a challenge to the field of oncology.

The cytoskeletal proteins of the cytokeratin (CK) family have been earlier reported to be associated with disease outcome and response to therapy. Whereas CK20 are found in the superficial umbrella cells, CKs 5, 6, and 14 are found in the basal cells[10–12] in normal bladder. Altered or anomalous expression of the CKs is indicative of aberrant differentiation in the process of urothelial carcinogenesis.[13,14] In fact, altered CK20/14 expression is correlated with poor prognosis.[11,15]

Even though it is now widely accepted that CKs' expression or their altered distribution affects both bladder cancer pathogenesis and disease outcome, the underlying mechanisms are not known. The *N*-butyl-*N*-(4-hydroxybutyl)

nitrosamine (BBN)-induced model is one of the most universally used model to study bladder carcinogenesis.[16,17] This model largely reminisces the multistep process that results in papillary urothelial neoplasms,[16,17] and allows in-depth study of the various steps of the process. In addition, the entire time span is short and thus feasible.[16,17] Hence, we decided to study CKs 14 and 18 expressions using this model.

Methods

Experimental design

Twenty female C57BL/6/c mice (Beijing Weitong Lihua Experimental Animal Technology Co., Ltd, Beijing, China) were housed under specific pathogen-free conditions before being divided into two groups ($n = 10$/group). Group 1 served as the control (no treatment, NT), which received only tap water. Group 2 was treated with BBN for 20 weeks following one week of no treatment. The BBN carcinogen (TCI America, Portland, OR) was administered *ad libitum* at 0.05% in drinking water to mice from eight to 20 weeks of age. Water consumption between groups was recorded to confirm equivalent BBN intake. All animal procedures were approved by the Institutional Animal Care and Use Committee of The Third Hospital of Hebei Medical University.

Histopathologic evaluation

For histopathology analysis, urinary bladders were excised, cut in longitudinal halves, and fixed in 10% buffered formalin. Formalin-fixed bladders were then paraffin embedded, sectioned, and stained with hematoxylin and eosin following standard protocols. Slides were histopathologically graded in a blinded fashion by two expert pathologist (Dr Feng Gao and Dr Yumei Ma), and bladders were categorized respectively as normal or cancerous, invasive or muscle invasive, bladders.

Immunostaining

Immunohistochemical and immunofluorescence analyses were performed as described before.[18] Imaging was performed using Nikon microscopy system. The NIS Elements software was used for semi-automated quantification of CK14+ (antibody from Convance PRB-155P) and CK18+ (Abcam ab668) cells. The slides were scored blinded to the identity of the specimen or staining type as percent of positively stained cells with a range of 0 to 100.

Western blotting

Lysates from tissue samples were made using RIPA buffer containing protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Shanghai, China). Fifty micrograms protein lysates were resolved by SDS-PAGE. Membranes were probed with anti-CK14 (ab9220) and anti-CK18 (ab82254) antibodies (Abcam, Waltham, MA). Each blot was also probed with anti-GAPDH antibody (Abcam) to confirm equal loading.

Statistical analyses

Statistical analyses were performed using SPSS version 20.0 (IBM Corporation, NY). Two-sided P values < 0.05 were considered statistically significant.

Results

Twenty female mice were divided into two groups—group 1 (NT) and group 2, which received BBN for 20 weeks plus one week without treatment. No lesions were observed in the control NT group (Figure 1(a)). Urothelial lesions were classified histologically as either hyperplastic/invasive (Figure 1(b)), or invasive throughout bladder (Figure 1(c)).

Following histological classification of bladder lesions, CK14 and CK18 immunostaining was assessed according to its distribution and intensity. In NT animals, basal cells and umbrella cells stained positive for CK14 (Figure 2(a) to (c)) and CK18 (Figure 3(a) to (c)), respectively. In comparison within the BBN group, hyperplastic lesions showed robust CK14 (75–100%) staining after 20 weeks (Figure 2 (d) to (f)). The CK14 staining in BBN group (mean percent score, 84.3 ± 9.2, median score 87) was significantly more intense and at higher proportions compared to the NT group (mean percent score, 16.7 ± 4.7, median score 12) ($P = 0.001$) (Figure 2(g)). In comparison within the BBN group, hyperplastic lesions showed robust CK14 (75–100%) staining after 20 weeks (Figure2(d) to (f)). In comparison, no increase of CK18 staining were observed in the BBN group (mean percent score, 1.9 ± 1.4, median score 2) compared to the NT group (mean percent score, 6.9 ± 3.7, median score 8) ($P = 0.0265$) (Figure 3(d) to (g)). The comparative increase in CK14 (Figure 4(a)) and decrease in CK18 (Figure 4(b)) expression in the BBN group animals

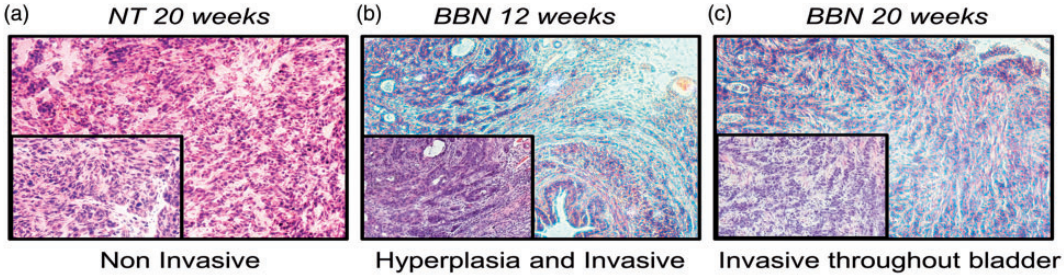
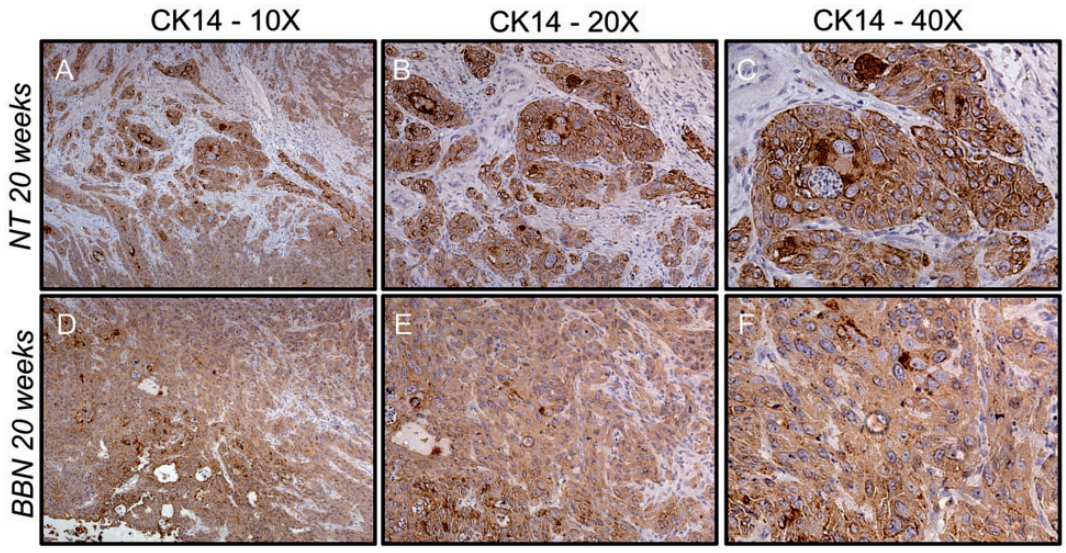


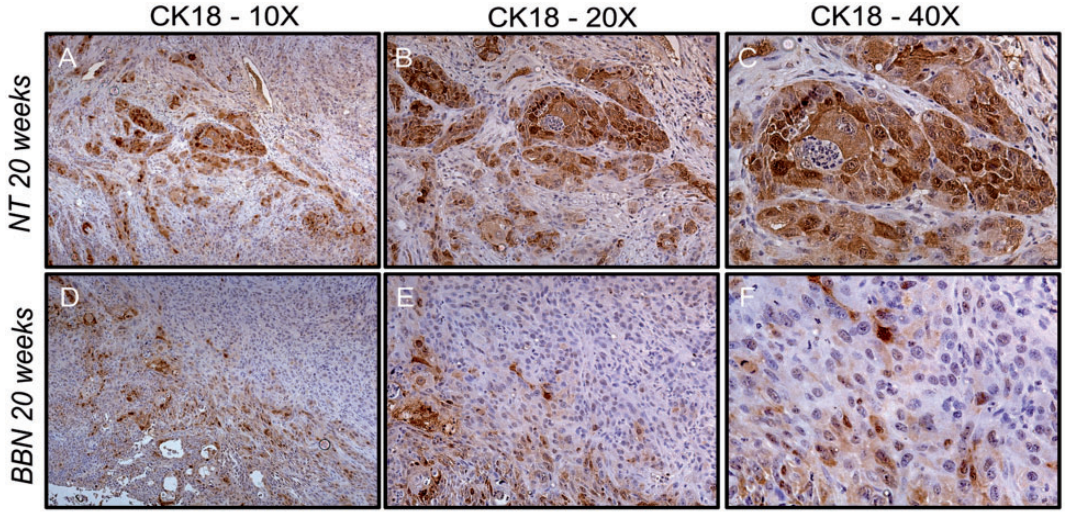
Figure 1. BBN treatment results in papillary bladder cancer in mice. Hematoxylin and eosin staining of bladder tissue isolated from mice with no treatment (NT) (a), BBN for 12 weeks (b), and BBN for 20 weeks (c). Images were obtained at 10× magnification. Inset shows images obtained at 40× magnification.
BBN: *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. (A color version of this figure is available in the online journal.)



G Cytokeratin 14

Group	N	Minimum	25th Pctl	Median	75th Pctl	Maximum
NT 20 weeks	10	0	3	12	15	20
BBN 20 weeks	10	75	78	87	95	100

Figure 2. CK14 expression is more pervasive in bladder cancer. Representative immunohistochemistry images obtained at 10× (a, d), 20× (b, e), and 40× (c, f) magnification showing CK14 staining in the no treatment (NT) (a–c) and BBN treated mice after 20 weeks (d–f). (g) Quantification of CK14 staining scores in the two groups. BBN: *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. (A color version of this figure is available in the online journal.)



G Cytokeratin 18

Group	N	Minimum	25th Pctl	Median	75th Pctl	Maximum
NT 20 weeks	10	0	1	8	12	18
BBN 20 weeks	10	0	1	2	3	5

Figure 3. CK18 expression is suppressed in bladder cancer. Representative immunohistochemistry images obtained at 10× (a, c), 20× (b, d), and 40× (c, f) magnification showing CK18 staining in the no treatment (NT) (a–c) and BBN treated mice after 20 weeks (d–f). (g) Quantification of CK18 staining scores in the two groups. BBN: *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. (A color version of this figure is available in the online journal.)

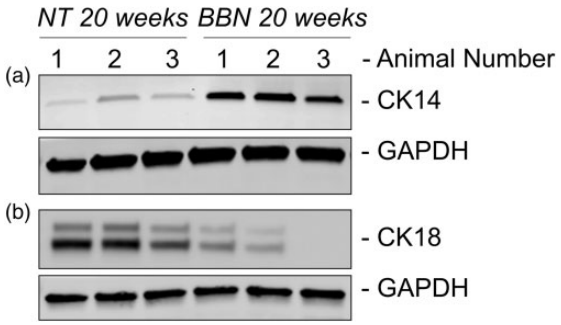


Figure 4. Immunoblot analyses corroborates immunohistochemical findings. Representative immunoblot images showing CK14 (a) and CK18 (b) expression in three animals each from the NT and BBN groups. Each blot was also probed with anti-GAPDH antibody to ensure equal loading among different samples. BBN: *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; NT: no treatment.

compared to NT group animals were confirmed by immunoblotting.

Immunofluorescence analysis showed that invasive carcinomas showed increased CK14 immunostaining in all epithelial layers (Figures 5(b), 6(a) and (b)) compared to the control NT group (Figures 5(a), 6(c) and (d)).

Discussion

Experimental modeling of bladder cancer pathogenesis is important to complement the data obtained in research with patient samples and cell lines, especially to better define the histogenesis of bladder cancer.[19,20] The urothelium can in fact be defined as a hierarchy of strict

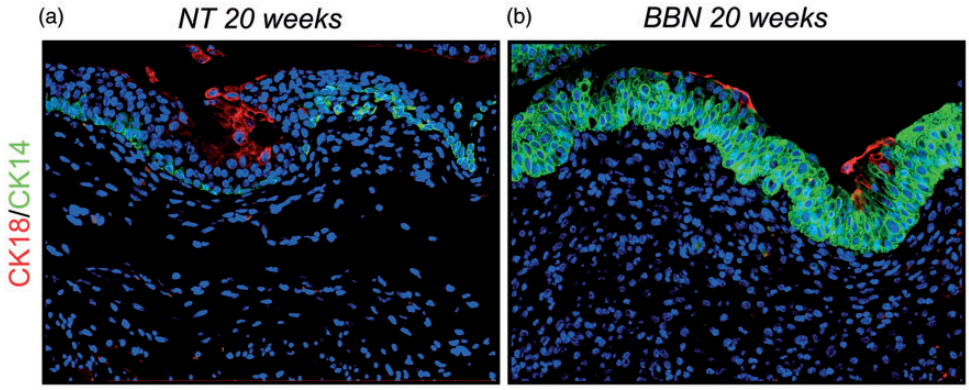


Figure 5. Invasive, high-grade papillary carcinomas showed increased CK14 immunostaining in all epithelial layers. Immunofluorescence images of CK18 (red) and CK14 (green) in representative bladder lesions in the no treatment (NT) and BBN-treated mice for 20 weeks (BBN). Scale bar: 100 μm.
BBN: *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. (A color version of this figure is available in the online journal.)

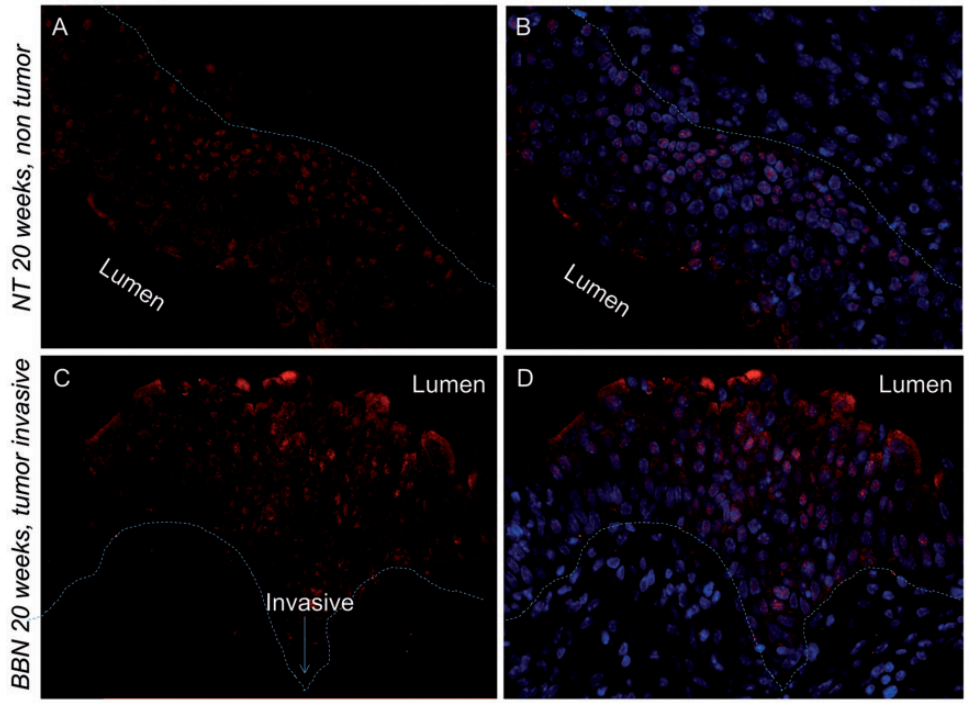


Figure 6. Invasive, high-grade papillary carcinomas showed increased CK14 immunostaining in all epithelial layers. Immunofluorescence images of CK14 (red) in representative bladder lesions in the no treatment (NT) (a, b) and BBN-treated mice for 20 weeks (BBN) (c, d). CK14 expression was detected both in the lumen and invasive fonts in the BBN group as opposed to just the lumen in the NT group. In (b) and (d), blue shows DAPI staining for nucleus. Scale bar: 20 μm.
BBN: *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; DAPI: 4',6-diamidino-2-phenylindole. (A color version of this figure is available in the online journal.)

developmental program regulated differentiation of multiple tissue-specific stem cell population.[12,21]

The BBN-induced bladder tumors in mice or rat provide an opportunity to study the entire cascade of bladder cancer histogenesis in an immunocompetent context.[17] In comparison, xenograft experiments using *in vitro* cell lines can be done only in the context of immunodeficient animals and thus does not allow the same depth or detail into the pathogenesis of bladder cancer.

In the context of BBN-induced bladder cancer model both mice and rat can be used, the only difference being that mice develop invasive lesions as opposed to papillary tumors in rats.[17] In the current study, we report for the first time the expression of CK14 and CK18 during the consecutive steps of BBN-induced carcinogenesis in the mice bladder. Interestingly, and perhaps importantly from a pathologist perspective, aberrant CK14 and CK18 expression was reminiscent of the early steps of neoplastic transformation.[17] Our results corroborate those of others indicating CK14 as one of the earliest marker of urothelial differentiation.[15,17]

It is not exactly known how CK14 expression is regulated in bladder cancer. There has been reports where genetic ablation of forkhead box A1 (*Foxa1*$^{-/-}$) led to high CK14 expression, [22] indicating that FOXA1 might be a transcriptional repressor of CK14 transcript. Sonic hedgehog signaling has also been indicated to be indirectly linked to CK14 expression in bladder cancer, even though nothing has been confirmed yet.[23] Hence, it will be important to define mechanisms underlying induction of CK14 expression in patients with bladder cancer.

One limitation of our study design was that experiments were conducted only in female mice; however, there is a well-known gender disparity in bladder cancer incidence, with higher incidence rates in males compared to females.[24] Both androgen and androgen receptor signaling are known to potentiate bladder carcinogenesis.[25,26] Similarly, there is evidence that suggests that the lower incidence risk in females is also due to the female sex steroids.[27–30] Hence, it will be determined if aberrant CK14 and CK18 expression pattern observed in females is mimicked in males with bladder carcinogenesis.

Given our findings and those of others,[15–18] it might be hypothesized that aberrant CK14 expression represents an early event of multistep carcinogenesis leading to bladder cancer. Future research will have to validate our observations in clinical samples from patients with bladder carcinoma and focus on cell lineage-tracing studies in animal models and patient samples that will further elucidate late steps of pathogenesis of bladder cancer.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; YPL, XPJ and YQJ conducted the experiments; WW supplied critical reagents; YLW, XLW and YXG wrote the manuscript. All authors have read and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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