The tumor-suppressor gene Nkx2.8 suppresses bladder cancer proliferation through upregulation of FOXO3a and inhibition of the MEK/ERK signaling pathway

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Invasive bladder cancer is a lethal disease for which effective prognostic markers as well as potential therapy targets are still lacking. Nkx2.8 (NK2 homeobox 8), a novel member of the NK-2 gene family, was found in a study of human neoplastic gene regulation (5). Some studies have suggested that Nkx2.8 plays an important role in human carcinogenesis by suppressing tumor formation in mice since the Nkx2.8-null mouse develops bronchial hyperplasia at birth, dysplasia in adulthood and invasive lung cancer at an advanced age (6). Moreover, an analysis of 14q13.3 (chromosomal location of Nkx2.8) in paired human lung cancer and adjacent normal tissues revealed that ~29% of the tumors have loss of heterozygosity, and most of those tumors express low levels of Nkx2.8 (7). Furthermore, overexpression of Nkx2.8 was shown to inhibit colony formation of H522 lung cancer cells (7). These findings suggest that Nkx2.8 acts as a tumor suppressor gene in lung cancer. However, the biological function of Nkx2.8 in the control of bladder tumorigenesis and tumor progression has not been characterized. In this study, we investigated the clinicopathologic significance and potential role of Nkx2.8 in the development and progression of bladder cancer.

Materials and methods

Cell lines

The bladder cancer cell lines T24 and 5637 were maintained in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT).

Paired tumor and tumor-adjacent tissues

Eight pairs of bladder cancer tissues and matched tumor-adjacent morphologically normal bladder epithelial tissues were frozen and stored in liquid nitrogen until used to compare the expression levels of Nkx2.8 protein and messenger RNA (mRNA).

RNA extraction, quantitative real-time PCR

Total RNA samples from cultured cells and primary tumor tissues were extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instruction. Quantitative real-time PCR (qRT–PCR) was performed as described previously (8). The primers were designed with Primer Express version 2.0 (Applied Biosystems, Carlsbad, CA) and are listed in Supplementary Table 1, available at Carcinogenesis Online. Relative gene expression was determined by normalizing to the geometric mean of the housekeeping gene GAPDH and calculated using the 2^-ΔΔCt method as described previously (9).

Western blotting and immunofluorescence analysis

Western blotting was performed as described previously (8) using anti-Nkx2.8 (Abcam, Cambridge, MA), anti-MEK, anti-phospho-MEK (Ser17/22), anti-ERK, anti-phospho-ERK (Thr185/Tyr186), anti-FOXO3a, anti-phospho-FOXO3a (Ser256), anti-cyclin D1, anti-Rb, anti-phospho-Rb (Ser780) and anti-p27 antibodies (Cell Signaling Technology, Danvers, MA). The membranes were stripped and re-blotted with a mouse monoclonal anti-α-tubulin antibody (Sigma, St Louis, MO) as a loading control.

Cells were stained for immunofluorescence analysis on coverslips as described previously (10). Briefly, the cells were incubated with primary antibodies against Nkx2.8 and then incubated with rhodamine-conjugated goat antibodies against rabbit IgG (Jackson ImmunoResearch Laboratories). The coverslips were counterstained with 4’-6-diamidino-2-phenylindole and imaged with a confocal laser-scanning microscope (Olympus FV1000). Data were processed with Adobe Photoshop 7.0 software.

Vectors and retroviral infection

The Nkx2.8 expression construct was generated by subcloning the PCR-amplified human Nkx2.8 coding sequence into the pBABE retroviral vector. To silence endogenous Nkx2.8, two small hairpin RNA (shRNA) oligonucleotides (RNAi#1: GCCGACGCTTCTCAGATTACC and RNAi#2: GCTTGGGCTTCTTTGATA TAT) were cloned into pSuper-retro-puro. Retroviral production and infection were performed as described previously (11). Based on their baseline expression of Nkx2.8, bladder cancer cells were either transduced with pBABE/Nkx2.8 or pSuper-retro-Nkx2.8-shRNAs. T24 cells showed no expression of Nkx2.8 and bladder cancer may lead to the identification of new prognostic markers and therapeutic targets.

Human Nkx2.8 (NK2 homeobox 8), a novel member of the NK-2 gene family, was found in a study of human neoplastic gene regulation (5). Some studies have suggested that Nkx2.8 plays an important role in human carcinogenesis by suppressing tumor formation in mice since the Nkx2.8-null mouse develops bronchial hyperplasia at birth, dysplasia in adulthood and invasive lung cancer at an advanced age (6). Moreover, an analysis of 14q13.3 (chromosomal location of Nkx2.8) in paired human lung cancer and adjacent normal tissues revealed that ~29% of the tumors have loss of heterozygosity, and most of those tumors express low levels of Nkx2.8 (7). Furthermore, overexpression of Nkx2.8 was shown to inhibit colony formation of H522 lung cancer cells (7). These findings suggest that Nkx2.8 acts as a tumor suppressor gene in lung cancer. However, the biological function of Nkx2.8 in the control of bladder tumorigenesis and tumor progression has not been characterized. In this study, we investigated the clinicopathologic significance and potential role of Nkx2.8 in the development and progression of bladder cancer.

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were infected with retroviruses carrying pBabe/Nkx2.8. The 5637 cells showed high expression of Nkx2.8 and were infected with retroviruses carrying pSuper-retro-Nkx2.8-shRNAs. Stable cell lines were selected with 0.5 μg/ml puromycin for 10 days. Cell lysates prepared from the pooled population of cells in sample buffer were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to confirm Nkx2.8 protein levels.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay
Cells (0.2 × 10⁶ per well) were seeded in 96-well plates. At each time point, the cells were stained with 100 μl sterile 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye (0.5 mg/ml; Sigma) for 4 h at 37°C, followed by removal of the culture medium and addition of 150 μl of dimethyl sulfoxide (Sigma). The absorbance was measured at 570 nm, with 655 nm as the reference wavelength. All experiments were performed in triplicate.

Anchorage-independent cell growth assay
Cells were trypsinized and counted. Five hundred cells were suspended in 2 ml complete medium plus 0.3% agar (Sigma). The agar-cell mixture was plated on top of a bottom layer with 1% complete medium agar mixture. After 10 days, viable colonies containing 50 cells or more >0.5 mm were counted. The assay was performed three times in independent experiments for each cell line.

Colony formation assay
Cells were plated on 60 mm plates (0.5 × 10⁵ cells per plate) and cultured for 10 days. The colonies were stained with 1% crystal violet for 30 s after fixation with 10% formaldehyde for 5 min.

Cell cycle phase analysis
Cell cycle phase analysis was performed using flow cytometry analysis as previously reported (12). In brief, all cells in a culture dish were harvested by trypsinization, washed in ice-cold phosphate-buffered saline and fixed in 80% ice-cold ethanol in phosphate-buffered saline. Before staining, the cells were spun down in a cooled centrifuge and resuspended in the cold. Bovine pancreatic RNAsér (Sigma–Aldrich) was added at a final concentration of 2 mg/ml, and the cells were incubated at 37°C for 30 min, followed by incubation in 20 mg/ml of propidium iodide (Sigma–Aldrich) for 20 min at room temperature. Fifty thousand cells per sample were acquired and analyzed on a flow cytometer (FACS Calibur; BD Biosciences, San Jose, CA).

Animal experiments
In total, 20 male BALB/c-nu mice (4–5 weeks of age, 18–20 g) were purchased from the Center of Experimental Animals of Guangzhou University of Chinese Medicine. The mice were housed in barrier facilities on a 12 h light/dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. Mice were randomly divided into four groups of five mice each. Cells (1 × 10⁶ T24 vector, 1 × 10⁶ T24-Nkx2.8, 5 × 10⁵ 5637 vector and 5 × 10⁵ 5637-Nkx2.8-RNAi#2) were suspended in RPMI 1640 medium and injected subcutaneously into the flank of mice into the skin of the mice. The tumor diameter was measured and the volume (length × width²/2) calculated every other day. Mice were humanely killed on day 28, and the tumors were dissected and weighed.

Patient information and tissue samples
This study included 161 patients with bladder cancer who had received radical cystectomy at the Sun Yat-sen University Cancer Center, Guangzhou, People’s Republic of China between 1 January 2000 and 30 December 2008. These patients had a median follow-up time of 32 months (range 6–104 months). The median time from surgery to tumor recurrence was 10 months (range 2–73 months). There were 38 patients with bladder cancer recurrence, including local recurrence alone in 9, distant recurrence alone in 9 and local recurrence with distant recurrence in 20 patients. The 161 paraffin-embedded radical cystectomy bladder cancer samples were analyzed by immunohistochemistry (IHC) to determine the relationship between Nkx2.8 expression and clinicopathological features and prognosis. Prior patients’ consent and approval from the Institutional Research Ethics Committee were obtained for the use of these clinical materials for research purposes. Clinical information on the samples is summarized in Supplementary Table 2, available at Carcinogenesis Online.

Tumor-node-metastasis staging was determined according to the 2002 American Joint Committee on Cancer tumor-node-metastasis classification of bladder cancer (13). Tumors were graded according to the World Health Organization 2004 guidelines (14).

IHC analysis
Nkx2.8 expression was determined by IHC on the 161 cases of paraffin-embedded bladder cancer tissues as described earlier (11,15) using a rabbit anti-Nkx2.8 antibody (1:800; Abcam). Normal goat serum was used as a negative control. The degrees of immunostaining of formalin-fixed paraffin-embedded sections were viewed and scored by two independent investigators who were blinded to the histopathological features and patient data of the samples. The scores given by the two independent investigators were averaged. Positive staining was scored as follows: 0, no positive tumor cells; 1, <20% positive tumor cells; 2, >20% positive tumor cells. The intensity of positive staining was scored according to the mean optical density (MOD) method (described previously and also briefly below) (16,17): 0, no staining; 1, weak staining (light yellow); 2, moderate staining (yellow brown) and 3, strong staining (brown). The staining index was calculated as the product of the proportion of positive cells and staining intensity score. An optimal cutoff value was identified based on a measurement of heterogeneity with a log-rank test for statistical analysis with respect to overall survival: staining index = 0 indicated negative Nkx2.8 expression, and an staining index score of ≥1 was indicative of positive Nkx2.8 expression. For the Ki-67 labeling index, the proportion of positive cells in the stained sections was evaluated at ×200 magnification, and the mean value of 10 representative fields analyzed from each section was recorded.

The MOD method was used for scoring the staining intensity in order to account for inconsistencies in IHC staining intensities. In brief, the stained slides were evaluated at ×200 magnification using the SAMBA 4000 computerized image analysis system with the Immuno 4.0 quantitation program (Image Products International, Chantilly, VA). Ten representative staining fields from each tumor sample were analyzed to determine the MOD (intensity of staining) by measuring the number of positive pixels within the whole tissue. A negative control with each batch of staining was used for background subtraction in the quantitative analysis.

Statistical analysis
All statistical analyses were carried out using the SPSS 13.0 statistical software package. The chi-square test and Fisher’s exact test were used to analyze the relationship between Nkx2.8 expression and clinicopathological characteristics. Bivariate correlations between study variables were calculated by Spearman’s rank correlation coefficients. Differences between average MOD values of different tissue groups were statistically analyzed using the t-test. Survival curves were plotted by the Kaplan–Meier method and compared using the log-rank test. Survival data were evaluated using univariate and multivariate Cox regression analyses. In all cases, P < 0.05 was considered statistically significant.

Results

Downregulation of Nkx2.8 in archived bladder cancer tissues
Nkx2.8 was markedly downregulated at both the transcriptional and translational levels in bladder cancer tissues compared with adjacent normal urothelial tissues by qRT–PCR and western blot, respectively (Figure 1A and B). Importantly, qRT–PCR analysis revealed that the normal/tumor (N/T) ratio of Nkx2.8 mRNA expression was as high as 45-fold in one case of paired primary bladder cancer tissues (Figure 1A). These results were further confirmed by IHC analysis, which also showed apparent downregulation of the Nkx2.8 protein in all eight bladder cancer tissues (Figure 1C).

Nkx2.8 inhibits proliferation of bladder cancer cells
To study the biological role of Nkx2.8 in bladder cancer progression, a stable Nkx2.8 expressing cell line T24/Nkx2.8 was established (Figure 2A). By western blotting, the Nkx2.8 protein was found to be highly expressed in the T24/Nkx2.8 stable cells, whereas it was not detected in the T24/vector control stable cell line. Immunofluorescence analysis also showed that the T24/vector cells did not stain for Nkx 2.8, whereas the T24/Nkx2.8 cells showed strong staining for Nkx 2.8 both in the cytoplasm and nucleus (Figure 2B). These results confirmed the specificity of the Nkx2.8 antibody used in the study.

In the MTT assay, T24/Nkx2.8 cells grew more slowly, with ~1.8-fold fewer cells than the T24/vector control cells by day 5 after plating (Figure 2C). The T24/vector cells also formed many more and larger colonies than those of the T24/Nkx2.8 cells in the colony formation assay (Figure 2D). The effect of Nkx2.8 on tumorigenic activity in bladder cancer cells was further demonstrated by the observation that its upregulation could significantly decrease the anchorage-independent growth of T24 cells, as indicated by the decrease in colony number and size on soft agar (Figure 2E). Furthermore, knockdown of endogenous Nkx2.8 in 5637 cells by shRNAs (Figure 3A) significantly decreased both cytoplasmic and nuclear Nkx 2.8 in 5637 cells (Figure 3B), and it...
Nkx2.8 regulates cell cycle factors cyclin D1 and p27kip1 through the MEK/ERK pathway in bladder cancer cells

Since the expression of Nkx2.8 appeared tightly linked to the G1/S phase transition of bladder cancer cells, we further investigated whether cell cycle factors, including the cell cycle promoter cyclin D1 and cell cycle inhibitor p27kip1, could be regulated by Nkx2.8. qRT–PCR analysis revealed that forced expression of Nkx2.8 led to a significant upregulation of p27kip1 and downregulation of cyclin D1 mRNA in T24 cells. In contrast, silencing of Nkx2.8 resulted in dramatic downregulation of p27kip1 and upregulation of cyclin D1 mRNA in 5637 cells (Figure 4C, upper panel). Western blot analysis confirmed that cyclin D1 and p27kip1 were regulated by Nkx2.8 at the translational level (Figure 4C, lower panel).

Because it has been documented that the expression of p27kip1 and cyclin D1 (18–20) can be transcriptionally regulated by FOXO3a, a downstream target protein of ERK (21–25), we hypothesized that Nkx2.8 can regulate the cell cycle through activation of the MEK/ERK/FOXO signaling pathway. As shown in Figure 4D, overexpression of Nkx2.8 significantly decreased the phosphorylation levels of MEK, ERK and FOXO3a, whereas no change in their total protein levels was observed. In contrast, inhibition of endogenous Nkx2.8 expression obviously increased the phosphorylated levels of MEK, ERK and FOXO3a. Interestingly, we also found that phosphorylation of the tumor suppressor Retinoblastoma protein was downregulated in Nkx2.8-overexpressing cells, whereas it was upregulated in Nkx2.8 silenced bladder cancer cells, with no changes in total Retinoblastoma protein levels.

Nkx2.8 inhibits xenografted tumor growth in vivo

Tumors formed from T24/Nkx2.8 cells implanted in nude mice grew more slowly and weighed substantially less than those formed by T24/vector cells after 28 days (Figure 2F). Furthermore, tumors derived from 5637 cells transduced with retroviruses expressing Nkx2.8-shRNA (RNAi#2) grew much faster and weighed significantly more at week 4 than those formed by 5637-vector cells (Figure 3F). Collectively, our results indicate that Nkx2.8 plays an important role in inhibiting the tumorigenicity of bladder cancer cells in vivo.

Correlation between Nkx2.8 expression and clinicopathologic features of bladder cancer

Whereas no Nkx2.8 staining was detected in the negative control (Supplementary Figure 1A and B is available at Carcinogenesis Online), strong Nkx2.8 staining could be observed mainly in the cytoplasm (Supplementary Figure 1C and D is available at Carcinogenesis Online). IHC analysis was then performed to determine the expression of Nkx2.8 in 161 paraffin-embedded archived bladder cancer tissues and 10 morphologically normal bladder epithelial tissues. Nkx2.8 expression was significantly decreased or not detected in bladder cancer tissues (Supplementary Figure 2A–C is available at Carcinogenesis Online). Among the bladder cancer tissues, 75.2% (121/161) of the cases showed positive expression of Nkx2.8, whereas the remaining samples (24.8%) had negative staining for Nkx2.8 (Table I). In contrast, Nkx2.8 was highly expressed in the adjacent non-cancerous tissues as well as normal bladder epithelial tissues. Quantitative analysis indicated significantly lower MODs of Nkx2.8 staining in T1–T4 primary tumors than in normal bladder epithelial tissues, with lower MOD values in tumors of higher T stages (P < 0.001, Supplementary Figure 2A and B is available at Carcinogenesis Online). We further analyzed the correlation between Nkx2.8 expression and clinicopathologic features of bladder cancer. As summarized in Table I, Nkx2.8 expression was significantly associated with T classification (P = 0.006), N classification (P = 0.017) and tumor multiplicity (P = 0.026). A significant correlation between the Ki-67 labeling index and Nkx2.8 expression in bladder cancer was also found (chi-square test,
Samples with negative expression of Nkx2.8 had a higher Ki-67 labeling index, whereas samples that were positive for Nkx2.8 expression had a lower Ki-67 labeling index (Supplementary Figure 2D is available at Carcinogenesis Online). These data were further confirmed by Spearman correlation analysis (Supplementary Table 3 is available at Carcinogenesis Online), and the coefficients for the correlations between Nkx2.8 expression and T classification, N classification, tumor multiplicity, and Ki-67 were 0.257 (P = 0.001), 0.222 (P = 0.017), 0.176 (P = 0.05), and 0.315 (P = 0.01).
Nkx2.8 expression is associated with good prognosis in bladder cancer patients

The cumulative 5 years survival rate of the Nkx2.8-positive group was 83.4% (95% confidence interval, 0.768–0.900), whereas it was only 40.5% (95% confidence interval, 0.253–0.577) in the Nkx2.8-negative group (log-rank test, \( P < 0.001 \), Figure 5A). In addition, multivariate survival analysis indicated that the Nkx2.8 expression, T classification and N classification were independent prognostic factors for outcomes in patients with bladder cancer (Supplementary Table 4 is available at Carcinogenesis Online).

Moreover, we analyzed the recurrence-free survival of patients who underwent adjuvant chemotherapy. Interestingly, we found that patients with negative Nkx2.8 expression had a much higher risk of recurrence than patients with positive Nkx2.8. As shown in Figure 5B, the 4 years recurrence-free survival rate was only 22.7% in the Nkx2.8-negative group, whereas it dramatically increased to 0.207 \( (P = 0.009) \), 0.179 \( (P = 0.023) \) and 0.198 \( (P = 0.012) \), respectively.

**Fig. 3.** RNAi-silencing of Nkx2.8 promotes cell growth and tumor-promoting activity. (A) RNAi-silencing of Nkx2.8 in shRNA-transduced stable 5637 cells. \( \alpha \)-Tubulin was used as a loading control. (B) Endogenous Nkx2.8 was stained both in cytoplasm and nucleus of 5637 cells, and Nkx2.8-shRNA significantly decreased both cytoplasmic and nuclear Nkx2.8. Silencing endogenous Nkx2.8 promoted cell growth as determined by (C) MTT assays, (D) colony formation assays and (E) anchorage-independent growth assays. Error bars represent mean ± SD from three independent experiments. (F) Tumor volumes measured on the indicated days. Data points are the mean tumor volume ± SD (left panel). Representative graph of tumor growth (middle panel) and mean tumor weights (right panel) 28 days after inoculation. * \( P < 0.05 \), ** \( P < 0.01 \).
61.5% in the Nkx2.8-positive group (log-rank test, \(P = 0.024\), Figure 5B).

Furthermore, the prognostic value of Nkx2.8 expression was analyzed when stratifying the patients based on tumor grade and T classification (pT). As shown in Figure 5C and D, the expression of Nkx2.8 was strongly associated with overall survival of patients with both low-grade tumor (Grade 1–2, log-rank test, \(P < 0.001\)) and high-grade tumor (Grade 3, log-rank test, \(P = 0.028\)). However, when it was examined according to T classification, the impact on the outcome associated with positive expression of Nkx2.8 continued to be more favorable only in the pT1–2 subset (Figure 5E, log-rank test, \(P = 0.001\)) but not in the pT3–4 subset (Figure 5F, log-rank test, \(P = 0.083\)).

**Discussion**

Nkx2.8 gene expression has been characterized in liver cancer and tracheobronchial stem cells (5,6). However, the protein expression of Nkx2.8 in human cancer tissue has only been reported in lung cancer (7). Our study reported, for the first time, the clinical significance of Nkx2.8 in bladder cancer. In this study, we found that Nkx2.8 was frequently reduced in primary bladder tumors. Furthermore, forced expression of Nkx2.8 in bladder cancer cell lines led to inhibition of proliferation and tumourigenicity *in vitro* and *in vivo*, accompanied with G1/S phase arrest, downregulation of cyclin D1, p-FOXO3a and upregulation of p27Kip1 as well as deactivation of the MEK/ERK pathway. Meanwhile, RNAi-silencing of Nkx2.8 induced converse results. The above results provided evidence that Nkx2.8 may play a potential tumor suppressor role in bladder cancer progression. In the present study, we use tumor adjacent morphologically normal bladder epithelium tissues as control. However, considering the high recurrence rate of bladder cancer, obtaining bladder epithelium from non-bladder cancer volunteers as normal controls may be helpful in future studies.

Negative expression of Nkx2.8 protein occurred in 24.8% of primary bladder tumors, which was significantly associated with advanced T and N classification, proliferation index and tumor multiplicity. Decreased or depletion of Nkx2.8 was correlated with poor prognosis and reduced survival of patients with bladder cancer. Multivariate analysis showed that loss of Nkx2.8 protein expression could be used as an independent prognostic predictor for bladder cancer patients. Specifically, we observed that the prognostic potential of the negative Nkx2.8 expression was only found in the pT1–2 subset but not in the pT3–4 subset. This finding illustrated that Nkx2.8 may play an important role in the initial phase of bladder cancer carcinogenesis. Thus,
Nkx2.8 expression in bladder uroepithelium tissue could aid in evaluating new cases of bladder cancer and guiding the follow-up schedule in bladder cancer patients. However, we should admit that using the negative expression of Nkx2.8 as a prognostic factor may be limiting due to its low incidence (24.8%).

Nkx2.8 is a homeodomain transcription factor usually located in the nucleus like other NK2-related homeobox proteins. For example, in mouse respiratory tract epithelium, Nkx2.8 is expressed in the nucleus (6). However, in our study, Nkx2.8 could be detected by IHC analysis in the cytoplasm in both normal urothelial and bladder cancer tissues, although at different levels. The cytoplasmic localization of Nkx2.8 was further confirmed in bladder cancer cells by immunofluorescence. By reviewing the literature, we found that altered intracellular localization of functional proteins is not uncommon. For example, b-catenin can play different roles in different intracellular locations, including the cell membrane, cytoplasm and cell nucleus (26,27). SAM68, a member of the RNA-binding protein family, normally located in the nucleus, has also been detected in the cytoplasm of renal cell carcinoma, breast cancer and cervical cancer tissues (8,28,29). More interestingly, SAM68 cytoplasmic localization is associated with clinicopathologic characteristics and prognosis. As Nkx2.8 can be detected both in the cytoplasm and nucleus of bladder cancer tissues and cells, we speculate that aside from being a transcription factor, it can also be cytoplasmically localized, which is not uncommon. For example, p27Kip1 and cyclin D1 by Nkx2.8 probably resulted from inhibition of the MEK/ERK/FOXO pathway. Interestingly, the level of phosphorylated Retinoblastoma was also dramatically suppressed by Nkx2.8. Taken together, these results explained the G1/S arrest phenomena caused by Nkx2.8. The G1/S arrest could prohibit G1/S phase transition by regulating expression of the cell cycle promoter cyclin D1 and inhibitor p27Kip1. We also demonstrated that Nkx2.8 inactivated the MEK/ERK/FOXO pathway by downregulation of p-MEK, p-ERK and p-FOXO3a. Therefore, the regulation of the pathways individually does not reveal significant prognostic power. Furthermore, lung cancer patients with co-activation of the thyroid transcription factor 1 and Nkx2.8 pathways appear to be resistant to standard cisplatin therapy (32). This observation suggests an oncogenic role of Nkx2.8 in lung cancer. On the other hand, some researchers have observed a tumor suppressor role of Nkx2.8 in lung cancer. The Nkx2.8-null mouse is known to develop invasive lung cancer (6), whereas another study showed that the Nkx2.8 loci is both amplified and deleted in lung cancer. Most tumors in certain subsets of NSCLC have low expression of Nkx2.8, and its overexpression can inhibit the growth of some lung cancer cells (7). Our study suggested that Nkx2.8 may play a tumor suppressive role in bladder cancer by inhibiting proliferation and growth of those tumor cells.

The molecular mechanisms underlying the potential role of Nkx2.8 in human cancer remain largely unknown. Herein, we showed that Nkx2.8 could inhibit the growth of some lung cancer cells (7). Our study suggested that Nkx2.8 may play a tumor suppressive role in bladder cancer by inhibiting proliferation and growth of those tumor cells.

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Table I. Correlation between Nkx2.8 expression and clinicopathological characteristics of bladder cancer patients

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<th>Characteristics</th>
<th>n</th>
<th>Nkx 2.8 Negative no. (%)</th>
<th>Nkx 2.8 Positive no. (%)</th>
<th>Chi-square test P-value</th>
<th>Fisher’s exact test P-value</th>
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<td>19 (63.3)</td>
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<tr>
<td>T4</td>
<td>19</td>
<td>8 (42.1)</td>
<td>11 (57.9)</td>
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<td>0.017</td>
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<tr>
<td>Low</td>
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<td>8 (18.6)</td>
<td>35 (81.4)</td>
<td>0.269</td>
<td>0.309</td>
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<tr>
<td>Unifocal</td>
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<td>22 (34.4)</td>
<td>42 (65.6)</td>
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<td>0.026</td>
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<tr>
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<tr>
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<td>24</td>
<td>5 (20.8)</td>
<td>19 (79.2)</td>
<td>0.622</td>
<td>0.799</td>
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<tr>
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<td>137</td>
<td>35 (25.5)</td>
<td>102 (74.5)</td>
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<td>&gt;50%</td>
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<td>27 (33.3)</td>
<td>54 (66.7)</td>
<td>0.012</td>
<td>0.017</td>
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<tr>
<td>&lt;50%</td>
<td>80</td>
<td>13 (16.3)</td>
<td>67 (83.8)</td>
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</table>

UIS, urinary irritation symptoms.
regulated by FOXO3a, which is a downstream target of ERK (18–24). Thus, our observations link Nkx2.8 to the basic cell cycle regulatory process and help provide evidence for diverse molecular mechanisms by which Nkx2.8 is able to inhibit proliferation and cell growth bladder cancer. However, the biological function and possible mechanism of Nkx2.8 in human cancer will need to be investigated in detail. Further studies designed to determine the regulatory mechanism of Nkx2.8 on the MEK/ERK/FOXO pathway are currently underway.

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Conflict of Interest Statement: None declared.

Fig. 5. Kaplan–Meier curves with univariate analyses (log-rank). (A) Patients with positive Nkx2.8 expression (bold line) had a cumulative 5 years survival rate of 83.4% (95% confidence interval, 0.768–0.900), compared with 40.5% for patients with negative Nkx2.8 expression (dotted line; 95% confidence interval, 0.253–0.577). (B) In patients who underwent adjuvant chemotherapy, the Nkx2.8-negative group had a much higher risk of recurrence compared to the Nkx2.8-positive group with 4 years recurrence-free survival rates of 22.7 and 61.5%, respectively. (C–F) Kaplan–Meier curves of overall survival stratified by Nkx2.8 status according tumor grade (C and D) and T classification (E and F). In both group of patients with low and high tumor grade, the overall survival was significantly longer in the Nkx2.8-positive group than in the Nkx2.8-negative group (C and D). When stratified according T classification, the significant difference of overall survival between Nkx2.8-positive and -negative tumors was only observed in early T classification (E) but not in late T classification (F).

References


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