Thioredoxin-interacting protein suppresses bladder carcinogenesis

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Thioredoxin-interacting protein (TXNIP), which has a tumor-suppressive function, is underexpressed in some human cancers. The function of TXNIP in vivo in carcinogenesis is not fully understood. Here, we show TXNIP to be downregulated in human bladder cancer according to grade and stage and also that loss of TXNIP expression facilitates bladder carcinogenesis using a mouse bladder cancer model. N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced bladder cancer was found in 100% of Txnip knockout (KO) mice at week 8 of 0.025% BBN administration but in only 22% of wild-type (WT) mice at the same point. Among growth stimulators, phospho-extracellular signal-regulated kinase (pERK) expression was stronger during bladder carcinogenesis in Txnip-KO mice than in WT mice. We then evaluated TXNIP’s effects on ERK activation through various growth stimulators and their receptors. Overexpression of TXNIP in human bladder cancer cells attenuated pERK expression upon stimulation with stromal cell-derived factor-1 (SDF-1) but not with epidermal growth factor or insulin-like growth factor-1. In Txnip-KO mice, immunohistochemical analysis showed enhanced expression of C-X-C chemokine receptor type 4 (CXC4), the receptor of SDF-1, and of pERK in urothelial cells during BBN-induced bladder carcinogenesis. Finally, subcutaneous injection of CXC4 antagonist, TF14016, attenuated pERK in urothelial cells and suppressed bladder carcinogenesis. These data indicate that TXNIP negatively regulates bladder carcinogenesis by attenuating SDF-1-CXC4-induced ERK activation. This signal transduction pathway can be a potent target in preventing or treating bladder cancer.

Introduction

Bladder cancer is the fifth most common malignancy in the Western society; >70 000 new cases of bladder cancer are diagnosed every year in the USA (1). About 70% of patients present with non-muscle invasive bladder tumors and the remaining 30% present with high-grade invasive tumors at initial diagnosis. Low-grade non-muscle invasive bladder tumors can be treated by transurethral resection with an excellent patient prognosis. But high-grade non-muscle invasive bladder cancer, including carcinoma in situ (CIS), tends to invade the muscle layer and metastasize (2), which causes high mortality or severe impairment of quality of life, even if it is cured. The development of bladder tumors is thought to be a multifactorial process and to occur as a direct consequence of the mutagenic actions of chemical or physical carcinogens, such as tobacco smoking. The precise mechanisms underlying carcinogen-induced bladder carcinogenesis have not yet been clarified. To approach this question, N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced mouse bladder carcinoma model has been used widely since the 1970s. One feature of this mouse model is that BBN can induce high-grade non-muscle invasive bladder cancer, followed by muscle invasive bladder cancer, in almost all subject animals (3).

In general, epithelial cells receive oncogenic growth signals that originate in the extracellular environment in response to carcinogenic signals (4). Aberrant activation of mitogen-activated protein kinase (MAPK) cascades, such as epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK) and oncogenic signals involving AKT have been shown to lead to excessive cell growth and division in early-stage carcinogenesis (5). Specifically, upregulation of oncogenic signaling genes involved in the Ras-Raf-MAPK cascade have been reported in the advanced stages of BBN-induced bladder cancer (6). The MAPKs are activated by receptor signaling pathways, including receptor tyrosine kinases [such as epidermal growth factor receptor (EGFR) and type 1 insulin-like growth factor receptor (IGF-1R)]. In addition, seven-transmembrane receptors have been also reported to activate MAPK signaling. Among the seven-transmembrane receptors leading to activation of MAPKs, C-X-C chemokine receptor type 4 (CXC4), which is activated exclusively by its ligand stromal cell-derived factor-1 (SDF-1), is upregulated in many kinds of cancers, including bladder carcinoma. Indeed, CXC4 was identified as the only chemokine receptor upregulated in muscle-invasive bladder cancer by screening expression levels of all chemokine receptors in normal urothelium and bladder cancer (7). It is also upregulated in high-grade non-muscle invasive bladder cancers including CIS (8), although the role of SDF-1-CXC4 signaling in bladder carcinogenesis remains unclear.

Thioredoxin-interacting protein (TXNIP), also known as thioredoxin-binding protein-2 or vitamin D3 upregulated protein 1 (VDUP1), is a multifunctional protein. It binds to and inhibits thioredoxin 1, a major redox-regulating molecule, and also plays roles in the development of natural killer cells, glucose metabolism, cell cycle arrest and inflammatory signal regulation (9–11). Regarding the relationship between TXNIP and cancer, TXNIP expression is downregulated in normal urothelium and bladder cancer (12). Furthermore, pharmacological blockade of SDF-1-CXC4 signaling inhibits vivo in vivo BBN-induced bladder carcinogenesis accompanied with marked downregulation of ERK. Here, we show the significant role of TXNIP on SDF-1-CXC4-ERK signaling.

Abbreviations: BBN, N-butyl-N-(4-hydroxybutyl) nitrosamine; CIS, carcinoma in situ; CXC4, C-X-C chemokine receptor type 4; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IGF-1R, type 1 insulin-like growth factor receptor; JNK, c-Jun N-terminal kinase; KO, knockout; MAPK, mitogen-activated protein kinase; pERK, phospho-extracellular signal-regulated kinase; SDF-1, stromal cell-derived factor-1; TXNIP, thioredoxin-interacting protein; VDUP1, vitamin D3 upregulated protein 1; WT, wild-type.
pathway in the molecular mechanisms underlying carcinogen-induced bladder carcinogenesis.

Materials and methods

Quantitative reverse transcription–polymerase chain reaction

Total RNA was extracted using an RNeasy Mini kit (QIAGEN, Germany) from cultured cells or whole bladders and reverse-transcribed into complementary DNA using the First-Strand cDNA Synthesis Kit (GE Healthcare, Piscataway, NJ). Following primers were used in reverse-transcription polymerase chain reactions (PCRs): human GAPDH (forward 5'-GAAGGTGAAGGTCGAGTC-3' and reverse 5'-GGGATTGGATGAGTTT-3'), human TNXP (forward 5'-GTGTTCAAGAAGAGACAG-3' and reverse 5'-CTCAGGGCTATACAAAGA-3'), mouse Gapdh (forward 5'-ACACAGTGCTGCTACATCACG-3' and reverse 5'-CTGCCACCTTGTGCTGTA-3'), mouse Txnip (forward 5'-TCTCCTAGAAGACGCGCTACAGG-3' and reverse 5'-CTCAGAACGG-3'). The real-time PCR reaction was performed using the 7300 Real-Time PCR System and reverse transcription–polymerase chain reaction (RT–PCR) reagents (Power SYBR Green PCR Master Mix; Applied Biosystems, Tokyo, Japan) according to the manufacturer’s protocol. The PCR reactions were done in triplicate; three independent experiments were performed using different sets of samples. Data were normalized relative to the expression level of GAPDH genes. Data in each group were compared with those in another group by post-hoc test protected least significant difference (Fisher’s PLSD); boxplot graphs were drawn using commercially available software.

Cell culture, stable transfectants and stimulation by SDF-1α, epidermal growth factor or IGF-1

Human bladder cancer cell lines: EJ, HT1376, J82, T24, KK47, RT112, RT4, 253J and TCCSUP were used. The cells were cultured in RPMI 1640 media supplemented with 1% fetal bovine serum. Stable 253J and TCCSUP transfectants overexpressing TXNIP were generated as described previously (18) with some modification. The cells were transfected with either pCMV-Tag2b control plasmid or pCMV-Tag2b containing the HA-tagged TXNIP (human TXNIP), human TXNIP, mouse TXNIP (forward 5'-TCTCCTAGAAGACGCGCTACAGG-3' and reverse 5'-CTCAGAACGG-3'). The expression of Tag2b or HA-TXNIP in the transfectants was confirmed using the corresponding rabbit or mouse IgG as negative controls for primary antibodies. Each tissue sample was observed by an eclipse E1000 microscope (Nikon, Tokyo, Japan). Western blot analysis

Total cellular proteins were extracted by disrupting the cells or homogenizing the whole bladders in RIPA buffer. Total cellular protein extracts from cultured cells, mouse bladder tissues and human bladder urothelium were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane, blocked with 1% non-fat dry milk and incubated with antibodies for the following proteins: HA (Covance Japan, Tokyo, Japan), CXCR4, β-actin (Abcam, Cambridge, UK), VDUP1, SDF-1 (Santa Cruz Biotechnology, Santa Cruz, CA), AKT, phospho-AKT (pAKT, ser473), JNK, phospho-JNK (pJNK, Thr183/Tyr185), ERK, pERK (Cell Signaling Technology Japan, Tokyo, Japan). Stained bands were detected using an enhanced chemiluminescence-advanced system (GE Healthcare Japan, Tokyo, Japan) and a LAS 4000 Mini analyzer and ImageReader software (Fujifilm, Tokyo, Japan). Densitometric scans of the stained bands were performed with ImageJ software (http://rsbweb.nih.gov/ij/). Data were normalized relative to the baseline value in each group. The differences in densitometric values for pERK, pAKT and pJNK between WT and Txnip-KO mice were determined by the Mann–Whitney U-test.

Immunohistochemistry of mouse bladder cancer

After BBN treatment, mouse bladders were inflated and fixed in 10% formalin. Mice without BBN treatment were used as negative controls. Human bladder cancer specimens obtained by transurethral cold cup biopsy were fixed in 10% formalin overnight. The tissues were embedded in paraffin and were stained with hematoxylin and eosin in accordance with standard procedures. The formalin-fixed and paraffin-embedded bladders were stained as described previously (8) with antibodies for VDUP1 or pERK. The specificity of the staining was confirmed using the corresponding rabbit or mouse IgG as negative controls for primary antibodies. Each tissue sample was observed by an eclipse E1000 microscope (Nikon, Tokyo, Japan).

Table 1. Characteristics of 39 bladder cancers and 6 normal urothelial samples including age, grade, sex, smoking habits and 5 years recurrence-free survival

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal urothelium</th>
<th>Bladder cancer</th>
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<tbody>
<tr>
<td></td>
<td>Low grade and stage</td>
<td>High grade and/or high stage</td>
</tr>
<tr>
<td>Patients (no.)</td>
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<td>12</td>
</tr>
<tr>
<td>Age (average (SD))</td>
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<td>66.3 (12.0)</td>
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<tr>
<td>Sex (% male)</td>
<td>83</td>
<td>75</td>
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<tr>
<td>Stage (%)</td>
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<tr>
<td>T4</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>% Tobacco smoker</td>
<td>33</td>
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</tr>
<tr>
<td>Briskman index (average (SD))</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>% 5 years recurrence-free survival</td>
<td>50</td>
<td>41*</td>
</tr>
</tbody>
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Briskman index: daily tobacco smoke times years.
*Cystectomy is performed followed by transurethral resection.
Treatment with CXCR4 antagonist TF14016

A potent CXCR4 antagonist, 4F-benzoyl-TN14003 (TF14016), was synthesized as described previously (20). TF14016 was dissolved with sterile water. TF14016 (7.5 mg/kg) was injected subcutaneously into nine each WT and Txnip-KO mice, every other day, in conjunction with 0.025% BBN administration. After 12 weeks of BBN administration, bladders were harvested 30 min after injection of TF14016 and analyzed using immunohistochemistry or western blot analysis.

Results

Txnip expression is downregulated in human bladder cancers and in the BBN-induced bladder cancer model

Expression of TXNIP mRNA in human bladder cancers and bladder cancer cell lines was analyzed by quantitative RT–PCR. As shown in Figure 1A, TXNIP mRNA expression was not evident in all seven bladder cancer cell lines examined and significantly decreased in human bladder cancers (n = 39) compared with normal urothelium (n = 6) (P < 0.0001). Its expression correspondingly decreased according to the grade and stage of the cancer (P = 0.026).

Next, the expression levels of Txnip mRNA were analyzed in BBN-induced mouse bladder cancers. Under our experimental condition, CIS started to develop after 8 weeks of BBN administration, and lesions were identified in 86% (six of seven) of the mice after 12 weeks of BBN administration. Submucosal invasion (pathological T1 or more) was identified in all mice (n = 5) at 20 weeks of BBN administration (Figure 1B). Over the course of the BBN-induced bladder carcinogenesis, Txnip expression declined time dependently and was reduced to ~24%, in average, of the baseline level at 20 weeks of BBN administration (Figure 1C). Interestingly, a 37% decrease in Txnip expression was observed at 4 weeks of BBN administration, at which no malignant changes were identified histologically. These data indicated that the expression of TXNIP gene was suppressed in the course of human and mouse bladder carcinogenesis.

KO of Txnip accelerates BBN-induced bladder carcinogenesis

To determine whether the decreased expression of Txnip contributes to the development of bladder cancer, the prevalence of bladder cancer in Txnip-KO mice was compared with that in WT mice, using the BBN-induced bladder carcinogenesis model. Bladder cancer was pathologically identified in 60% (three of five) and 100% (seven of seven) of the Txnip-KO mice at 4 and 8 weeks of BBN administration,

Fig. 1. mRNA level of TXNIP gene is suppressed in bladder cancers. (A) Compared with normal urothelium, TXNIP is suppressed in human bladder cancers. Particularly in high-grade and/or high-stage (pT1 or more) cancers, TXNIP is strongly suppressed. All bladder cancer cell lines express little if any TXNIP. (B and C) Txnip is suppressed in a BBN-induced bladder cancer model. Bladder CIS developed after 8–12 weeks of BBN administration in WT mice (B). (C) Sequential change of mRNA of Txnip in BBN-induced mouse bladder carcinogenesis. Txnip is suppressed at 4 weeks of BBN administration and declined time dependently over the course of BBN administration; n = 3 in each group. Statistical analysis: Post-hoc test (Fisher’s protected least significant difference). (D) Western blot for TXNIP in BBN-induced mouse bladder carcinogenesis. TXNIP is downregulated at 8 weeks of BBN consumption and downregulated over the course of BBN administration.
Fig. 2. *Txnip*-KO mice develop BBN-induced bladder cancer earlier than WT mice. (A) Representative histologies of mouse bladder in WT mice (top) and *Txnip*-KO mice (bottom). Hematoxylin and eosin staining, original magnification ×200 (NCT) and ×100 (8 and 12 weeks). (B–D) Histograms showing ratio of bladder cancer according to duration of BBN treatment. Overlaid line indicates mean *Txnip* levels of WT mice at each time point relative to the baseline. (B) Overall cancers; (C) high-grade cancer; (D) invasive cancer (pT1 or more) and/or SCC. (E) Representative immunostaining for TXNIP in WT mouse bladder cancer. TXNIP level in bladder urothelia was downregulated in CIS at 12 weeks and T1 cancer at 20 weeks of BBN treatment compared with baseline (0 week) in WT mice (Figure 2D).
respectively, whereas it was identified in none of five and only 22% (two of nine) of the WT mice at 4 and 8 weeks, respectively (Figure 2A and B). When focusing on high-grade CIS, Txnip-KO mice developed this type of bladder cancers ~4 weeks earlier than WT mice (Figure 2C). Moreover, invasive bladder cancer (pT1 or more) and/or squamous cell carcinoma, which have characteristic histological features to advanced BBN-induced mouse bladder cancers, were observed also ~4 weeks earlier in the Txnip-KO mice than in the WT mice (Figure 2D). Thus, knocking-out Txnip accelerated BBN-induced bladder carcinogenesis, suggesting that TXNIP plays a suppressive role for bladder carcinogenesis. Although bladder carcinogenesis in WT mice is delayed, it is not prevented and is similar to the carcinogenesis in WT mice at 16–20 weeks of BBN treatment. This is probably because by that time, TXNIP levels were similar in the two groups, as shown in Figure 2E. The TXNIP level in bladder urothelium was downregulated at 12 and 20 weeks of BBN treatment compared with baseline in WT mice (n = 3 in each group, Figure 2E).

**Phosphorylation of ERK is enhanced in Txnip-KO mouse bladders during BBN-induced bladder carcinogenesis**

To investigate the molecular mechanisms underlying bladder carcinogenesis in Txnip-KO mice, the activation levels of oncogenic growth stimulatory signals were analyzed during BBN-induced bladder carcinogenesis in the WT and Txnip-KO mice. The phosphorylation levels of AKT, JNK and ERK were upregulated at 8 weeks compared with baseline (0 week) in both WT and Txnip-KO mice (n = 3 in each group, Figure 3A). Among them, upregulation of pERK was ~4-fold enhanced in Txnip-KO mice compared with WT mice at 8 weeks of bladder carcinogenesis (P < 0.05), whereas there were no obvious differences in the upregulation of pAKT and pJNK between Txnip-KO and WT mice. Immunohistochemical analysis demonstrated that the staining for pERK was enhanced in urothelial cells after 8 weeks of BBN administration in Txnip-KO mice (Figure 3B). Thus, loss of Txnip resulted in enhanced activation of ERK in bladder urothelium as well as accelerated BBN-induced bladder carcinogenesis.

**Overexpression of TXNIP attenuated SDF-1/CXCR4-induced phosphorylation of ERK in human bladder cancer cells**

To investigate the impact of TXNIP on ERK activation induced by various growth stimulations, such as EGF, IGF-1 or SDF-1, three stable transfectants with HA-tagged TXNIP were established from 253J and TCCSUP bladder cancer cell lines (253J-HA-TXNIP-1, 2 and TCCSUP-HA-TXNIP). Western blot analysis revealed that phosphorylation levels of ERK upon SDF-1 stimulation was lower in 253J-HA-TXNIP-1, -2 and TCCSUP-HA-TXNIP than in control cell lines (Figure 4A and B and supplementary Figure S1 is available at Carcinogenesis Online). On the other hand, there were no differences in pERK upon EGF or IGF-1 stimulation between 253J-HA-TXNIP-2 and control cells (Figure 4C). Similar results were observed in the other two stable transfectants (253J-HA-TXNIP-1 and TCCSUP-HA-TXNIP) upon EGF or IGF-1 stimulation (data not shown). There were also no differences in the expression levels of corresponding receptors, including EGFR, IGFIR, CXCR4, phospho-EGFR or phospho-IGF-1R (data not shown). These results indicate that overexpression of TXNIP attenuates SDF-1-CXCR4-induced activation of ERK exclusively.

**CXCR4 antagonist TF14016 suppresses pERK and BBN-induced carcinogenesis in Txnip-KO mice**

The expression status of SDF-1, CXCR4, ERK and pERK were assessed in BBN-induced bladder carcinogenesis. SDF-1 and ERK were constantly expressed until 8 weeks of BBN administration, whereas CXCR4 was gradually upregulated in the course of bladder carcinogenesis in the WT mice. When Txnip-KO mice were compared with WT mice, there were no obvious differences in the expression of SDF-1 and CXCR4. On the other hand, the expression of pERK was more enhanced in Txnip-KO mice than in the WT mice (Figure 5A).

To assess the impact of activation of ERK induced by SDF-1-CXCR4 stimulation on enhancement of bladder carcinogenesis in Txnip-KO mice, SDF-1-CXCR4 signaling was blocked by a specific CXCR4 antagonist TF14016 in both WT mice and Txnip-KO mice. TF14016 was injected subcutaneously into WT mice and Txnip-KO mice every other day for 12 weeks during BBN administration. No significant adverse effects, including weight loss, were observed due to TF14016. Western blot analysis demonstrated that TF14016 markedly attenuated pERK in the mouse bladder of both groups to the similar level after 12 weeks of BBN consumption (Figure 5B). Pathological examination revealed that the prevalence of malignant bladder lesion was remarkably lower in TF14016-treated WT mice and Txnip-KO mice than in each control mice (Figure 5C and D).

**Discussion**

The present study shows novel findings that expression of Tnnip mRNA is suppressed in human bladder cancers and in a mouse model of BBN-induced bladder carcinogenesis. Moreover, we have clearly demonstrated that loss of Tnnip facilitates BBN-induced mouse bladder carcinogenesis using Tnnip-KO mice. In general, a tumor suppressor gene is defined as a gene whose partial or complete inactivation...
Fig. 4. (A) Forced expression of TXNIP attenuates pERK response to SDF-1 in human bladder cancer cell lines. The cell line 253J-HA-TXNIP-1 and -2 showed decreased activation of pERK after SDF-1 treatment compared with the control cells (arrowhead). Similar results were obtained from TCCSUP-HA-TXNIP. Densitometric values are shown in the chart below. (B) Timeframe analysis of pERK activation after SDF-1 treatment in fetal bovine serum-free medium. Lower panel shows time-dependent changes in densitometric values for pERK. Similar results were obtained from TCCSUP-HA-TXNIP. (C) Forced expression of TXNIP did not influence major receptor tyrosine kinase pathways including EGFR and IGF-1R signaling.

Fig. 5. (A) Western blot for SDF-1, CXCR4 and ERK in BBN-induced bladder carcinogenesis of both WT and Txnip-KO mice. CXCR4 is induced at 2 weeks of BBN administration in both WT and Txnip-KO mice, whereas the SDF-1 level did not show obvious changes. On the other hand, pERK was more enhanced in Txnip-KO mice than in the WT mice. (B–D) A specific CXCR4 antagonist TF14016 suppressed BBN-induced ERK activation and bladder carcinogenesis in Txnip-KO mice. (B) TF14016 suppressed pERK after 12 weeks of BBN administration in both WT mice and Txnip-KO mice. (C) Representative histology of mouse bladder. TF14016 suppressed bladder carcinogenesis after 12 weeks of BBN administration in both WT mice and Txnip-KO mice. Original magnification ×200. (D) TF14016 reduced the incidence of bladder carcinogenesis after 12 weeks of BBN administration in Txnip-KO mice to almost the same level as observed in WT mice.
leads to an increased likelihood of cancer development (4). Txnip is thought to be a tumor suppressor gene based on observational evidence of suppressed expression in cancer tissues from various origins (12–15) and functional verification using cultured cancer cells in *in vitro* experimental series (9–11). Reportedly, Txnip-deficient mice are also predisposed to renal (21) and hepatocellular (22,23) carcinomas. Our data, which indicate that Txnip functions as a tumor suppressor in urothelium, are consistent with its decreased expression in several kinds of cancers.

We have investigated *in vivo* functional consequences of TXNIP loss using a BBN-induced bladder carcinogenesis model in the Txnip-KO mice. In general, carcinogenic stimuli, such as BBN, boost oncogenic growth-stimulatory signals, leading to excessive cell growth and division at the early stage of carcinogenesis (5). Therefore, we investigated ERK, JNK and AKT signaling as major growth-stimulatory signals and found that ERK was more active in Txnip-KO mice than in WT mice. Abrerrant activation of ERK, a second messenger involved in an important oncogenic signal transduction pathway, is reportedly associated with bladder carcinogenesis, among other cancers. On the other hand, some possible mechanisms of TXNIP have been listed in tumor suppression. A previous *in vitro* study reported that TXNIP decreases cell proliferation by restricting cell cycle progression; TXNIP deficiency reduced expression of p27, a cyclin-dependent kinase inhibitor (24). Alteration of redox status has been also proposed as a mechanism of tumor suppression since TXNIP inhibit the activity of thioredoxin 1. However, under the conditions we used to induce bladder carcinogenesis, we could not find obvious differences in the expression of thioredoxin 1, cell cycle-related genes including p27 and the status of redox [8-hydroxy-2′-deoxygenosine (8-OHdG)] between Txnip-KO mice and WT mice (data not shown). Collectively, TXNIP can function as a tumor suppressor mainly through regulating ERK activity, which is strongly associated with oncogenic process toward bladder carcinogenesis. Since the present study remains observational, the precise mechanism of action should be clarified in further studies.

Phosphorylation of ERK can occur via activation of either of several signal pathways including receptor tyrosine kinases (i.e. IGF-1R and EGFR) or seven-transmembrane receptors (i.e. CXCR4). In the present study, BBN administration induced both CXCR4 expression in urothelial cells and SDF-1 expression in stromal mesenchyme cells. In contrast, bladder cancer cell lines transfected with Txnip-KO demonstrated attenuated CXCR4-ERK activation upon SDF-1 stimuli. Therefore, CXCR4 is considered to be a major upstream receptor activating ERK signaling; it plays an important role in bladder carcinogenesis by stimulating several transcription factors. Indeed, CXCR4 antagonist significantly attenuates ERK activation and suppresses bladder carcinogenesis in both WT and Txnip-KO mouse models to almost the same level. Since the effects of TXNIP deletion are abrogated by the CXCR4 antagonist, there seems to be a causal link between TXNIP deletion and enhanced bladder carcinogenesis. These results suggest that TXNIP suppression facilitates bladder carcinogenesis by enhancing CXCR4-ERK activation.

Another important upstream regulator of ERK is the Ras signaling pathway, which is activated by receptor tyrosine kinases (i.e. IGF-1R, EGFR and FGFR3. Abrerrant activation of Ras oncoproteins is associated with development of low-grade non-muscle invasive bladder cancer (25). However, BBN-induced mouse bladder cancer shows a morphologic effect similar to another bladder carcinogenesis pathway, namely high-grade CIS muscle-invasive cancer pathway (25). In the present study, restoration of TXNIP did not alter ERK activation upon IGF-1 or EGFR stimuli and CXCR4 blockade suppressed BBN-induced carcinogenesis. Also, TXNIP expression is significantly more suppressed in high-grade muscle-invasive human bladder cancers than in low-grade non-muscle-invasive types of cancer. These findings suggest that activation of SDF-1-CXCR4 signaling drives a CIS muscle-invasive bladder cancer pathway and that a major effect of TXNIP loss is to facilitate this pathway. Recent reports support the notion that the ERK pathway plays an important role in muscle invasive bladder cancer (26–29). Because TXNIP expression is also suppressed in low-grade non-muscle-invasive bladder cancers compared with normal urothelia, loss of TXNIP may play other roles in bladder carcinogenesis, which should be elucidated in future studies.

Using a BBN-induced bladder carcinogenesis mouse model, we have shown that CXCR4 antagonist TF14016 can reduce bladder carcinogenesis. The 14-mer peptide T140 was developed from structure–activity relationship studies of two antibacterial and antiviral peptides: 17-mer peptide tachyplesins and 18-mer peptide polyphemusins isolated from the Japanese horseshoe crab (*Tachypleus tridentatus*) and the American horseshoe crab (*Limulus polyphemus*), respectively. T140 possesses potent anti-HIV activity by binding specifically to CXCR4 and TF14016 is a potent and biostable T140 analog (20). To our knowledge, this is the first report of successful bladder cancer prevention using a drug that attenuates a specific oncogenic signal. A limitation of the present study is that the therapeutic effect of TF14016 on established bladder tumors have not been clarified. Therefore, it seems the most reasonable and practical to use this drug to prevent recurrence of high-grade bladder cancer after transurethral resection. Prevention of post-transurethral resection bladder cancer recurrence is a major clinical challenge, although some anticancer drugs and Bacillus Calmette-Guérin are currently available with partial effects. Although some newly developed antigen-based drugs and molecular-targeted agents have been tested for this purpose (30), none of these novel treatments have been established so far. Peptide-based TF14016 and its analogs also offer advantages in terms of cost over other novel treatment modalities. Although we did not find any lethal reaction or significant weight loss during the 12 weeks’ treatment, further evaluation of acute and chronic adverse events is essential for the future clinical use.

In conclusion, TXNIP, which is silenced during bladder carcinogenesis, suppresses ERK activation in the SDF-1-CXCR4 signaling and also bladder carcinogenesis, suggesting that SDF-1-CXCR4-ERK signaling might be a good target to prevent bladder cancer.

**Supplementary material**

Supplementary Figure S1 can be found at http://carcin.oxfordjournals.org/

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**References**


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