Tenascin-C promotes bladder cancer progression, depends on syndecan-4 and involves NF-κB signaling activation

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Abstract

Bladder cancer (BCa) is an unfortunately critical genitourinary tract disease with an uncertain pathology. Increasing evidence indicates that the tumor microenvironment is decisive with respect to cancer progression, and that this is driven by tumor cell interactions with stromal components. Tenascin-C (TN-C) is an important extracellular matrix (ECM) component and TN-C has been reported to be involved in other cancers, i.e. breast cancer. Expression of TN-C in BCa tissue is reported to positively correlate to BCa pathologic grade, yet the presence of urine TN-C is regarded as an independent risk factor for BCa. Thus, we assessed the value of TN-C in BCa tissues and noted that it also was increased according to tumor grade and was an independent risk factor for BCa. In fact, TN-C contributes to BCa cell migration, invasion and proliferation and this is dependent on syndecan-4 and involves NF-κB signaling activation. How syndecan-4 is linked to activation of NF-κB signaling is unclear. Our data provide a foundation for future investigations into TN-C's contribution to BCa progression.

Introduction

Bladder cancer (BCa) accounts for 90–95% of urothelial carcinomas and is the most common urinary tract malignancy[1]. Almost 80% BCas initially are diagnosed as non-muscle invasive BCa (NMIBC) which have better prognosis, but some of these tumors will progress to muscle invasive BCa (MIBC). Even with surgical interventions, 30% of BCas become invasive[2] and worsen patient prognosis[3]. The remaining 20% of BCas are MIBC at initial diagnosis and have less favorable prognosis-5% of patients have metastatic BCa[4]. Complete resection of all tumor tissue by transurethral bladder tumor resection (TURBT) is recommended for NMIBC, followed by chemotherapeutic instillation[5]. However, for special types of BCa, such as T_1G_3 or Carcinoma *in situ* (CIS), special treatments are available. Radical cystectomy (RC) with extended lymphadenectomy is considered the standard treatment for MIBC[6], followed by cisplatin-based adjuvant chemotherapy. Two different pathological pathways[7,8]are thought to contribute to MIBC and this is responsible for different prognoses between initially diagnosed MIBC and MIBC that is derived from NMBIC[9]. Therefore, understanding how BCa progression occurs is needed to establish better BCa therapy.

The tumor cell microenvironment differs from normal tissue[10-13], and contributes to cancer progression. Cancer cell interplay with stromal components, such as fibroblasts, macrophages, fibronectin, and the initiation of fibrosis is thought to be needed for tumor recurrence, drug-resistance, and poor prognosis[12,14-16]. A vital component of the extracellular matrix (ECM) in tumor cells, tenascin-C (TN-C) may have multifaceted and complicated roles in tumor progression.

TN-C is large (~300 kDa) as an intact monomer and ~1800 kDa when assembled as a hexamer[17]. After an initial identification in gliomas in 1983, TN-C has since been noted to appear in head and neck squamous cell carcinoma, breast cancer[18], prostate[19], thyroid[20], and pancreatic cancers[21], melanomas,

gastric cancer[17], and osteosarcomas[22], and in most of these cancers, TN-C is considered to be a tumor promoter that worsens prognosis.

TN-C is thought to operate in the surrounding tumor microenvironment by binding to its receptor annexin II[23-25] or its co-receptor syndecan-4/α5β1[26-29], and causing loss of focal adhesions and mitogenesis and increased migration[30, 31]. How binding to other receptors initiates subsequent functions is not established [32-34].

Four syndecan family members are found in mammals, and of these three (syndecan-1, 2 and 3) have a restricted tissue distribution. Syndecan-4 is expressed ubiquitously and is a member of the membrane-intercalated proteoglycans [28,35]. Binding to fibronectin within two independent sites with syndecan-4 and α5β1 is key to homeostasis of normal tissue[36], and involves activation of downstream signals related to cytoskeletal organization and cell proliferation. TN-C is reported to compete with the binding site of fibronectin with syndecan-4 and this interaction with syndecan-4 partially destroys the effects of this co-receptor, as well as attenuates the interaction of syndecan-4 with fibronectin, enhancing tumor cell malignancy. This process also includes FAK and Rho signaling[36].

Activation of NF-κB signaling, manifested by the nuclear translocation of P65 [37,38] was demonstrated with IHC staining in BCa tissues and this is reported to positively correlate to tumor progression. EMT progression is another aspect of this signaling[39,40], promoting tumor malignancy. Previous work suggests that TN-C is crucial to cancer progression[17] and urinary TN-C may be a useful biomarker of BCa progression[41-43].

To assess this idea, we used IHC to quantify TN-C expression in BCa tissues and noted that it was elevated with worsening tumor grade and was associated with shorter survival. Thus, TN-C expression is an independent risk factor for BCa patients. Additional studies suggest that TN-C promotes BCa cell line migration, invasion and proliferation via NF-κB signal activation that depends on syndecan-4.

Results

TN-C expression in BCa tissue increases with tumor grade and is an independent risk factor for BCa

TN-C expression in tumor, such as breast cancer, is negatively associated with survival and is an independent risk factor[44], but this relationship is unconfirmed in other tumors[17]. We noted that urinary TN-C is an independent risk factor for BCa in the presence of other limited conditions (such as exclusion of inflammation) and these data agree with published studies[41]. Whether TN-C expression in BCa tissues in local Chinese patients is critical is uncertain although its expression has been noted in BCa patients of other regions[45]. To understand this significance, we measured TN-C expression in BCa tissues from Chinese patients. **Figures 1 A, B show that** TN-C expression across different grades of BCa tissue differs and increases with severity of tumor grade. In addition, we observed that BCa tumor grade was an independent risk factor for BCa patients (**Fig. 1 C**). To understand TN-C expression and patient survival,

patients were stratified as high- or low-TN-C expressers. **Figure 1 D indicates that** TN-C expression exceeding the mean suggests poor survival and these data agree with published information[46]. Finally, a correlation analysis suggested that TN-C expression in BCa tissue is negatively correlated to tumor-free survival (**Fig 1 E**).

Preparation of stable high- and low-TN-C expressing cell lines

Previous results indicate that TN-C expression may drive BCa progression, so we measured TN-C in BCa cell lines from different tumor grades. We know that TN-C is secreted into the ECM and we measured TN-C in cell media to monitor this secretion. Data indicate diverse expression of TN-C in BCa cell lines (**Fig. 2 A, B**), and that T24 and J82 had higher TN-C expression and 5637 and 253J had lower TN-C expression. ELISA data agreed as did Western blot and real time PCR results, suggesting that TN-C may function as a secreted protein (**Fig. 2 C**).

To understand how TN-C contributes to BCa progression, we silenced TN-C expression in BCa cell lines and monitored malignancy and proliferation. Briefly, T24 and J82, TN-C-positive cell lines, were used and TN-C was knocked down with shRNA/Sc. Then, 5637 and 253J, TN-C-negative cell lines were manipulated to overexpress TN-C with a plasmid as mentioned in ***Material and Methods.*** TN-C-knock down or TN-C overexpression data are in **Figure 2 D**, **E and F**, indicating successful preparations.

TN-C enhances migration, invasion and proliferation in BCa cell lines

TN-C contributes to tumor migration, invasion and proliferation in diverse tumors, including breast cancer[47], melanoma, and pancreatic cancer. Our data show that TN-C is vital to BCa progression. We knocked down TN-C in T24 and J82 cell lines and noted reduced cancer cell migration and invasion (**Fig. 3 A, up**), attenuated proliferation (**Fig. 3 B, up**) and delayed and prolonged wound healing (**Fig. 3 C, up**). TN-C overexpression in 5637 and 253J cells enhanced migration and invasion (**Fig. 3 A, down**), increased proliferation (**Fig. 3B, down**) and accelerated wound healing (**Fig. 3C, down**).

Research suggests a complicated role for TN-C as an ECM component, but our TN-C staining data from BCa tissues indicates that ECM deposition of TN-C occurs beyond the cytoplasm. Thus, tumor cells exposed to exogenous TN-C may be modified. We added human TN-C peptide to media of TN-C-negative cell lines ($T24^{siTN\text{-}C}$, $J82^{siTN\text{-}C}$, 5637^{Vec}, and $253J^{Vec}$) as well as added human TN-C neutralizing antibody to TN-C-positive cell lines ($T24^{Sc}$, $J82^{Sc}$, $5637^{TN\text{-}C}$, and $253J^{TN\text{-}C}$) and a Boyden chamber assay, a wound healing study and BrdU incorporation was assessed to measure malignancy.

Exogenous TN-C (*Ex* TN-C, *Ex*) enhanced migration, invasion, and proliferation of 5637^{Vec} (**Fig. 4 A, B, C: 5637^{Vec}, Con *Vs Ex***), and knock down of TN-C attenuated T24 cell migration, invasion, and proliferation and this could be rescued with exogenous TN-C (**Fig. 4 A, B, C: $T24^{siTN\text{-}C}$, Con Vs *Ex***). TN-C neutralizing antibody added to media decreased migration, invasion and proliferation of $T24^{Sc}$ (**Fig. 4 A, B, C: $T24^{Sc}$, Con Vs Anti**) and $5637^{TN\text{-}C}$ (**Fig. 4 A, B, C: $5637^{TN\text{-}C}$, Con Vs Anti**) cells.

TN-C contributes to elevated expression of EMT-related markers and expression of MMP_2/MMP_9 by activating NF-κB signaling

Our data show that TN-C promotes BCa cell line migration, invasion, and proliferation, but how this happens is uncertain. TN-C may execute this function as a component of the ECM, at least partially. In transitional cell carcinoma, enhanced migration and invasion of tumor cells is often accompanied with epithelial to mesenchymal transition (EMT)[48], and this can be used to monitor malignant behavior of BCa cells. Activation of NF-κB has been causally linked to an invasive phenotype and can directly or indirectly induce expression of Snail, Slug, Twist, Zeb1, and Zeb2[49], all of which are markers of EMT. Thus, we hypothesize that TN-C-induced effects may involve NF-κB signaling. Data from Western blot and real time PCR to monitor expression of EMT-related markers, and immunofluorescent staining to quantify NF-κB signaling confirmed data from the Boyden chamber assay and wound healing studies. Also, knock down data for TN-C with T24 cells confirmed decreased expression of MMP_2/MMP_9, vimentin, N-cadherin and Snail, and this was accompanied by elevated expression of E-cadherin (**Fig. 5 A, B, $T24^{Sc}$-Con *Vs* $T24^{siTN-C}$-Con**), which is a reversal of EMT.

Moreover, this process was reversed in 5637 cells overexpressing TN-C which caused elevated expression of MMP_2/MMP_9, vimentin, N-cadherin and Snail but decreased expression of E-cadherin (**Fig. 5 A, B, 5637^{Vec}-Con *Vs* 5637^{TN-C}-Con**). The effect of knocking down TN-C in T24 cells could be reversed with *Ex* TN-C (**Fig. 5 A, B, $T24^{siTN-C}$-Con *Vs* $T24^{siTN-C}$-*Ex***), and TN-C neutralizing antibody significantly inhibited expression of these genes in $T24^{Sc}$ cells (**Fig. 5 A, B, $T24^{Sc}$-Con *Vs* $T24^{Sc}$-Anti**). Similar data were obtained with 5637 cells; *Ex* TN-C (**Fig. 5 A, B, 5637^{Vec}-Con *Vs* 5637^{Vec}-*Ex***) promotes EMT, and TN-C neutralizing antibody inhibits this (**Fig. 5 A, B, 5637^{TN-C}-Con *Vs* 5637^{TN-C}-Anti**).

Also, TN-C induced activation of NF-κB signaling. P65 is the functional subunit of the NF-κB dimer (P65/P50), and nuclear translocation of this subunit is thought to activate this signaling. **Fig. 5 C shows that** TN-C induces nuclear translocation in $T24^{siTN-C}$ *Vs* $T24^{Sc}$ cells and 5637^{Vec} *Vs* 5637^{TN-C} cells, just like $T24^{siTN-C}$-Con *Vs* $T24^{siTN-C}$-*Ex* and 5637^{Vec}-Con *Vs* 5637^{Vec}-*Ex*. However, nuclear translocation is inhibited by TN-C functional inhibition (**Fig. 5 C, $T24^{Sc}$-Con *Vs* $T24^{Sc}$-Anti, 5637^{TN-C}-Con *Vs* 5637^{TN-C}-Anti**).

TN-C induced activation of NF-κB signaling depends on syndecan-4

TN-C chiefly functions as a component of ECM, indicating an interaction between TN-C and tumor cells and syndecan-4 is reported to be the receptor involved in those interactions[50]. Briefly, syndecan-4 is regarded as a co-receptor of syndecan-4/α5β1, which is important to cell adhesion. Interference with this co-receptor causes tumor cell proliferation and metastases, so TN-C functions may depend on this membrane receptor in BCa cell line. To assess this, we measured expression of syndecan-4 in all BCa cell lines and **Fig. 6 A shows that** significant syndecan-4 expression was observed in all lines. There was no apparent difference among cell lines. Thus, T24 and 5637 cells were selected to represent TN-C-positive

and negative-cell lines, respectively and we studied the role of syndecan-4 by knocking down its expression with siRNA.

Two TN-C related stable cell clones, $T24^{Sc/siTN\text{-}C}$ and $5637^{Vec/TN\text{-}C}$ cells were also treated to knock down syndecan-4 expression and study the effect of TN-C on that expression. Data show that syndecan-4 expression was knocked down in both cell lines but knocking down of TN-C did not change syndecan-4 expression (**Fig. 6 B**).

To determine whether syndecan-4 knock down in both cell lines could modify migration and invasion, a Boyden chamber assay was employed and syndecan-4 knock down was noted to decrease migration and invasion in both cell lines. Effects of overexpression of TN-C (in 5637 cells) or the exogenous TN-C addition (in $T24^{siTN\text{-}C}$ and 5637^{Vec} cells) were attenuated (**Fig. 6 C**).

Also, syndecan-4 interference inhibits P65 nuclear translocation, blocking signal activation as shown by immunofluorescent staining (**Fig. 6 D**). Effects of overexpression of TN-C (in 5637 cells) or addition of *Ex* TN-C (in $T24^{siTN\text{-}C}$ and 5637^{Vec} cells) were blocked as well. Thus, TN-C enhances cancer cell line migration, invasion and proliferation, as well as activation of NF-κB signaling and this depends on syndecan-4.

Discussion

High recurrence of BCa is likely attributed to interactions of tumor cells with the surrounding microenvironment to drive progression, metastasis and drug resistance. Macrophages from prostate cancer tissue can induce cancer phenotypes of normal prostate epithelial cells when co-cultured[51]. Also, fibroblasts, inflammatory cells, and the ECM have vital roles in cancer, among which, TN-C is the least understood.

TN-C is reported to be important to embryogenesis, inflammation, and wound healing, and behaves in a similar manner in tumorigenesis. TN-C expression in cancerous tissue has been documented in various tumors and is regarded to be an independent risk factor for cancer patients. Consistent with the literature, TN-C expression data for BCa tissue in our study suggests a positive role in BCa progression, but TN-C expression across different BCa cell lines is diverse and does not correlate with tumor grade. For example, in 5637 cells (ATCC®HTB-9™, *www.atcc.org*) TN-C expression is not observed in grade II BCa, this may be explained by different sources of TN-C. In BCa cell lines cancer is the sole TN-C source, but tumor cell secretions or mesenchymal cells can also produce TN-C.

Previous reports suggest a vital role of TN-C in tumor progression and TN-C content in BCa cell lines is consistent with tumor cell TN-C expression. (**Fig. 2 A, C**). Modifying TN-C expression in BCa cell lines causes the same effect to TN-C concentration in the corresponding BCa cell lines (**Fig. 2 D, F**). Thus, secreted TN-C may be a primary source of TN-C tumor activity. TN-C neutralizing antibody reduces TN-C overexpression, as does human TN-C peptide (**Fig. 4**). Thus, in BCa cell lines, TN-C executes its role

mainly as a component of ECM, perhaps by binding with membrane receptors. Investigations have confirmed that the co-receptor of syndecan-4/α5β1 is important to tumor cell adhesion to fibronectin of ECM, and that interfering with this by TN-C decreases tumor cell adhesion and enhances metastasis and proliferation. Syndecan-4 is the sole syndecan family member that is ubiquitously expressed in the cell membrane. Many downstream signals of syndecan-4 are known, including PKCα, PKCδ, PI3K/Akt, and synectin[29,52]. Previous reports suggest that interference with syndecan-4/α5β1 co-receptor in the cell membrane inhibits normal cell proliferation and enhances tumor cell proliferation, but why this happens is not certain [26,27].

We found that knocking down syndecan-4 expression attenuates TN-C-induced tumor migration, invasion and proliferation, suggesting that TN-C contributes to metastasis and proliferation in a manner that depends on syndecan-4. However, how TN-C binds to membrane syndecan-4 and how this is connected to metastasis and proliferation is not known. Forced expression of TN-C or in the presence of *Ex* TN-C up-regulates mesenchymal markers (elevated expression of vimentin, Snail, N-cadherin, MMP_2/MMP_9) and decreases expression of E-cadherin **(Fig. 5 A, B)** in BCa cell lines, indicating that alternative expression of these genes may be related to the activation of NF-κB signaling.

We offer evidence that in BCa cell lines, activation of the NF-κB signal leads to EMT, manifested as previously depicted[53]. Immunofluorescent staining confirmed binding of TN-C, either by forced expression or exogenous TN-C, with syndecan-4 induces nuclear translocation of P65, a process that can be inhibited by syndecan-4 knock down (**Fig. 6 D**). Thus, binding of TN-C with syndecan-4 induces NF-κB signal activation and promotes tumor cell metastasis and proliferation.

Our present work is summarized in **Figure 7.** NF-κB is the downstream of the PI3K/Akt pathways, suggesting that TN-C binding with syndecan-4 may induced activation of the NF-κB signal is involved in PI3K/Akt pathway activation via the cytoplasmic domain of syndecan-4. Whether binding of TN-C with syndecan-4 involves the co-receptor α5β1 is unknown, but we suggest that TN-C promotes tumor cell metastasis and proliferation and this depends on syndecan-4. These data offer a solid foundation for future studies into the role of TN-C in BCa progression and may be a potential therapeutic target for treating BCa.

Materials And Methods

Tissue preparation and patient follow up

BCa tissue samples (N = 57) were obtained from the Department of Urology, at the First Affiliated Hospital of Xi'an Jiaotong University (32 males; age range 39–78 years-of-age; mean 63.7 ± 7.5 years). Pathological grading was monitored by three independent hospital pathologists and 15, 18, and 24 samples of grade I–III were noted, and all were transitional cell carcinomas. Samples were fixed in 4% formalin and paraffin-embedded.

To assess any TN-C expression and tumor grade correlation, TN-C and survival time was assessed and patients who gave the tumor samples were contacted by telephone. Survival was a normal distribution as demonstrated by Shapiro-Wilk test. This study was approved by the Ethics Committee of Xi'an Jiaotong University.

Immunohistochemical (IHC) staining of TN-C in BCa tissues

IHC was performed with a Dako Autostainer Plus system (Dako Corporation, Carpinteria, CA). Tissues were de-paraffinized, rehydrated and subjected to 5-min pressure-cooking antigen retrieval, 15-min endogenous enzyme block, 60-min primary antibody (1:300) incubation and 30-min DakoCytomation EnVision-HRP reagent incubation with rabbit antibodies (1:200). Signals were measured according to substrate hydrogen peroxide using DAB as a chromogen followed by hematoxylin counterstaining. Negative controls were prepared by omitting primary antibody. Stained (brown) cells were quantified by counting the positive cells X 100/total cells in 10 random microscopic (400X) fields in each slice.

Cell culture

Human BCa cell lines 5637, T24, RT4, J82, 253J, UM-UC-3 were obtained from ATCC (Manassas, VA), RPMI-1640 (for 5637) and DMEM (for the other cell lines) were from Invitrogen, (Carlsbad, CA), and medium was supplemented by 10% FBS (Invitrogen). Cells were cultured under 5% CO_2 at 37 °C. (Incubators: Thermo-scientific, location, Germany).

Real-time PCR

Total RNA was isolated from frozen tissues and cell lines using Trizol reagent (Invitrogen) and quantified by reading the absorbance at 260 nm. RNA (2 μg) was reverse transcribed using Revert Aid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocol.

For real-time PCR, we used the SYBR® *Premix Ex Taq*™ II system (TaKaRa Biotechnology Co., Ltd, Dalian, China) and a Bio-Rad CFX96™ Real-time system (Bio-Rad, city, CA). Then, 12.5 μl SYBR® *Premix Ex Taq*™ II, 1 μl primer (10 μM, primers; Table 1), 200 ng cDNA and 9.5 μl double de-ionized water were mixed. Then, pre-degeneration was conducted at 95 °C, 30 sec, for one repeat, and PCR was carried out at 95 °C for 5 sec followed by a 60 °C incubation for 30 sec, and 35 repeats. Next, dissociation was carried out at 95 °C for 15 sec followed by a 60 °C incubation for 30 sec, and another 95 °C incubation for 15 sec and data collection. GAPDH was a loading control.

Western blot

Cells are harvested when 80% confluent and washed with cold PBS three times. Total cellular protein lysates were prepared with RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate] containing proteinase inhibitors (1% Cocktail and 1 mM PMSF, Sigma, St Louis, MO). Protein (30 μg) was separated with 6% (for TN-C) or 10% (for others) SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked at room temperature for 1 h with 5% skim milk in

Tris-buffered saline without Tween 20 (pH 7.6, TBS). Polyclonal antibody TN-C was applied (1:300 dilution; Table 2) with 5% skim milk in TBS at 4 °C overnight, followed by washing with TBST (with Tween 20, pH 7.6), and membranes were incubated with secondary antibodies (Licor, Rockford, IL) coupled to the first antibody at room temperature in the dark for 1 h. Then, membranes were washed in the dark room, dried with neutral absorbent paper, and scanned using an Odyssey detection system (Licor). GAPDH was a loading control.

Boyden chamber assay and wound healing assay

Migration and invasion were tested with a Boyden chamber assay (Millipore, city, Switzerland). For the migration assay, 0.2 ml FBS-free DMEM medium suspension with 1 x 10^4 cells was added to the upper chamber and then 0.8 ml FBS-free DMEM was added to the lower chamber. After 24 h incubation, chambers are washed with PBS (pH 7.4) three times to remove attached cells in the upper chamber. Prior to staining (25 min) with crystal violet (0.01% in ethanol), cells were fixed with 4% formalin for 15 min and washed three times. Crystal violet-stained cells were under an inverted microscope and five cell count observations were randomly taken under a 200× objective, and cell counts were averaged. For the invasion assay, suspension in the upper chamber contained 0.2 ml FBS-free DMEM/Matrigel=8/1 (Matrigel, Sigma) and 1 × 10^4 cells were incubated for 36 h. The cells were treated as in the migration assay.

Wound healing was assessed by scratching 6-well dishes with a 10 μl pipette tip when cells were 80% confluent. Scratch widths were compared at 0, 12, and 36 h.

Preparing stable clone cell lines

PsiCHECK-2^{TNC} plasmids (Addgene plasmid 26995, http://www.addgene.org) and a vector were transfected into 5637 and 253J (TN-C negative) BCa cell lines, respectively. Lipofectamine^{TM} 2000 (Life Technologies, city, state) was used for transfection according to kit instructions and stable cell clones highly expressing TN-C were selected quantified with Western blot and real time PCR.

Short hairpin RNA (shRNA/Sc) to knock down TN-C expression in T24 and J82 cells (TN-C positive) was measured. pGPU-6-GFP$^{TN\text{-}C/Sc}$ was transfected into cell lines as mentioned above, low-TN-C expressing lines were chosen using G418 (600 μg/ml), and Western blot and real time PCR was used to assess shRNA effects. siRNA to knock down syndecan-4 expression was used (Table 1). A protocol for transfection using Lipofectamine^{TM} 2000 is the same as mentioned above.

BrdU incorporation assay

BrdU was added to cell media (3 μg/ml) after cells reached 60–70% confluence on coverslips and incubated for 4 h. Then, coverslips were rinsed three times with PBS for 10 min to remove free BrdU and samples were fixed in 4% paraformaldehyde for 45 min, followed by rinsing five times with PBS for 20 min. Then, 0.1% Triton X-100 was added to destroy the cell membrane (15 min) and 2N HCl (25 min) was

added to separate DNA into single strands for primary antibody access to incorporated BrdU. Before blocking nonspecific epitopes with 10% BSA for 20 min, cells were rinsed three times with PBS for 10 min to remove HCl and Triton. Then 10% BSA with anti-BrdU antibody (1:200) was added and incubated overnight at 4 °C.

The next day, cells were rinsed five times with PBS to remove free antibody, and cells were incubating with TRTIC-labeled second antibody for 1 h at room temperature and rinsed another three times with PBS to remove free antibody. Fluorescent intensity of TRITC was monitored with a Super Micro Orifice Plate Spectrophotometer (BioTek, city, state) at 547 nm.

ELISA

TN-C in cell media was measured with ELISA. Briefly, cell line (different groups) media were collected and tested with ELISA analysis according kits instructions (Human TN-C ELISA Kit, Shanghai Westang Biological Technology Co., Ltd. Shanghai, PRC), and optical density was measured at 450 nm. Data are expressed in μg/ml.

Immunofluorescent staining for nuclear translocation of NF-κB

Prepared cells were washed three times in cold PBS (pH = 7.4) and fixed with 4% paraformaldehyde for 15 min, followed by permeabilization in 0.5% Triton X-100 for 10 min and blocking with 1% BSA for 1 h. Rabbit anti-human-P65 in 1% BSA was added to media and incubated overnight at 4 °C. Mouse anti-rabbit TRITC (Red) IgG antibody (Santa Cruz, city, CA) diluted 1:100 in blocking buffer was added to media and incubated 1 h. Then cells were washed with cold PBS three times and cell nuclei were stained with DAPI (10 μg/ml, Sigma) for 3 min. Cells were observed under an Image Pro Plus System mounted on a fluorescent microscope (Olympus, Japan).

Other reagent and experiments

TN-C peptide (Millipore, location) was an exogenous TN-C added to media (1 μg/ml), and TN-C neutralizing antibody (1 μg/ml) (MAB2138, Novus, location*)* was used to neutralize TN-C in the media.

Statistical analysis

Statistical analysis was performed with a SPSS 15.0 statistic package (Chicago, IL). For comparisons among different grades, one-way ANOVA was used, and for comparisons between two different grades, The Student's t-test was used. A Shapiro-Wilk test was used to confirm distribution of survival time of BCa patients. P-values <0.05 were considered to be statistically significant.

Declarations

Author contributions statement

- Guan zhenfeng: Conceptualization, Methodology, Validation, Writing-Review & Editing, Visualization, Supervision, Project administration, Funding acquisition
- Sun Yi:Formal analysis, Investigation，Resources;
- Jiang Yazhuo: Data Curation, Writing-Original Draft, Methodology
- Mu Liang，Data Curation，Methodology
- Fan Jinhai: Conceptualization, Validation , Writing-Review & Editing ,Funding acquisition

Additional information

The author(s) declare no competing interests.

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Tables

Table1:Primers for Real-time PCR and siRNA/shRNA

Gene ID	Gene		Primers
NM_001145138.1	*P65*	F	ACG AAT GAC AGA GGC GTG TAT AAG G
		R	CAG AGC TGC TTG GCG GAT TAG
NM_002046.4	*GAPDH*	F	AAC AGC GAC ACC CAT CCT C
		R	CAT ACC AGG AAA TGA GCT TGA CAA
NM_004360.3	*E-Cadherin*	F	TGC CCA GAA AAT GAA AAA GG
		R	GTG TAT GTG GCA ATG CGT TC
NM_001792.3	*N-Cadherin*	F	ACA GTG GCC ACC TAC AAA GG
		R	CCG AGA TGG GGT TGA TAA TG
NM_003380.3	*Vimentin*	F	GAG AAC TTT GCC GTT GAA GC
		R	GCT TCC TGT AGG TGG CAA TC
NM_005985.3	*Snail1*	F	ACC CCA ATC GGA AGC CTA ACT
		R	GGT CGT AGG GCT GCT GGA A
NM_004530.4	*MMP2*	F	CTC ATC GCA GAT GCC TGG AA
		R	TTC AGG TAA TAG GCA CCC TTG AAG A
NM_004994.2	*MMP9*	F	TGA CAG CGA CAA GAA GTG
		R	CAG TGA AGC GGT ACA TAG G
NM_002999.3	*Syndecan-4*	F	CCA GTT TGA TGT TGC TGG GTG GTT
		R	AGC CCT AGA GCC TGA AGA AAG CAA
	Syndecan-4 siRNA	Si	5 -AAG GCC GAT ACT TCT CCG GAG-3
		Sc	5 -AAG GCT CTC CGG AGC GATA CT-3
NM_002160.3	*Tenascin-C*	F	AGC TTC CAA GAA ACA CCA CTT C
		R	CCA TCC CAG CCA ACC TCA
	Tenascin-C shRNA	F	5'-CAC CGC ACC TGA AGG CCT GAA ATT CTT CAA GAG AGA ATT TCA GGC CTT CAG GTG CTT TTT TG-3'
		R	5'-GAT CCA AAA AAG CAC CTG AAG GCC TGA AAT TCT CTC TTG AAG AAT TTC AGG CCT TCA GGT GC-3'

Table2: information of the antibodies

Gene ID	Antibody	Dilutions	Species	Supplied by
NM_004360.3	E-Cadherin	1:600	homo	*Santa Cruz*
NM_001792.3	N-Cadherin	1:300	homo	*Santa Cruz*
NM_003380.3	Vimentin	1:300	homo	*Santa Cruz*
NM_005985.3	Snail1	1:400	homo	*Santa Cruz*
NM_001145138.1	P65	1:300	homo	*Santa Cruz*
NM_004530.4	MMP2	1:400	homo	*Santa Cruz*
NM_004994.2	MMP9	1:400	homo	*Santa Cruz*
NM_002046.4	GAPDH	1:15000	homo	*Abcam*
NM_002999.3	Syndecan-4	1:400	homo	*Santa Cruz*
NM_002160.3	Tenascin-C (For WB)	1:300	homo	*Santa Cruz*
	Tenascin-C (peptide)		homo	*Millipore*
	Tenascin-C (For functional blocking)		homo	*NOVUS*

Figures

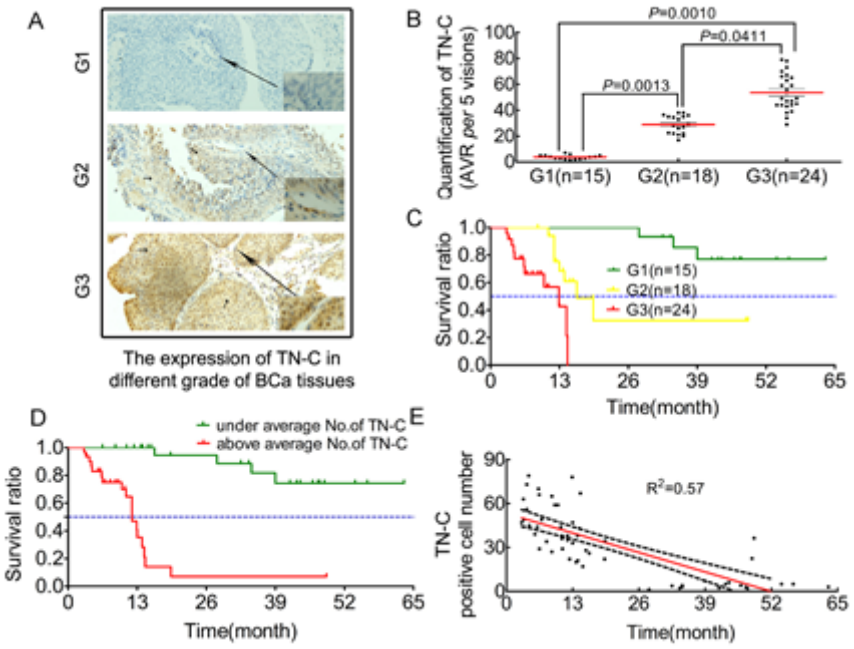

Figure 1

TN-C expression in BCa tissue and clinical significance. A. Representative figures of IHC staining for TN-C in different BCa tissues; Black arrow: the positive cell; B. quantification of IHC staining for TN-C in different BCa tissues, indicating elevated expression of TN-C according to BCa tumor grade, $P < 0.05$; C. Kaplan-Meier analysis suggests that tumor grade is an independent risk factor for BCa; D. Kaplan-Meier analysis indicates that TN-C expression in BCa tissue is an independent risk factor for BCa, based on normal survival distribution demonstrated by the Shapiro-Wilk test. Patients are stratified by average TN-C positive cells: green line indicates below average; red line indicates above average, $P < 0.05$; E. Correlation analysis suggests that tumor-free survival is negatively associated with TN-C expression in BCa patients, $P < 0.05$.

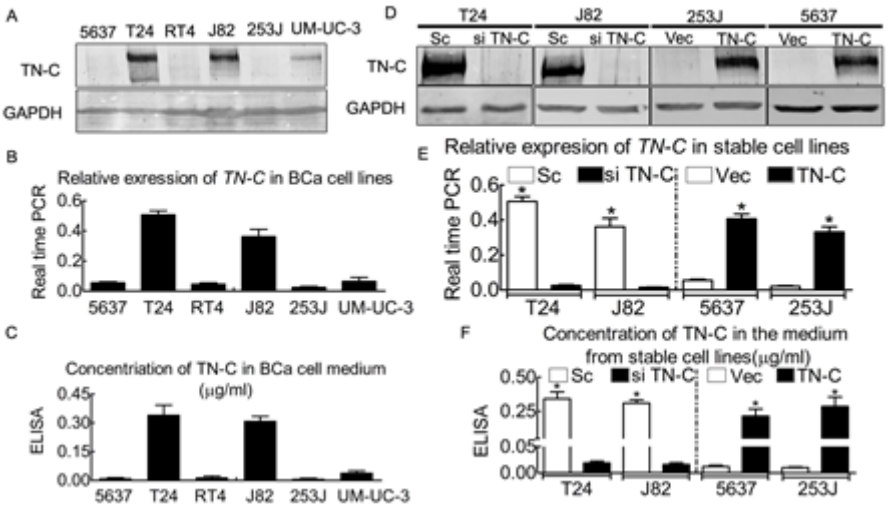

Figure 2

TN-C expression in BCa cell lines. A. Western blot of TN-C expression in BCa cell lines; GAPDH was a loading control, indicating different expression profile, with the strongest expression for T24 and J82, accompanied by the weakest for 253J and 5637; B. Real time PCR indicates TN-C expression in BCa cell lines, $*P < 0.05$, suggesting expression differences; C. ELISA depicting TN-C in BCa cell line medium, $*P < 0.05$, consistent with protein and mRNA; D. Western blot indicates efficiency of TN-C knockdown (T24si TN-CVs T24sc and J82si TN-C Vs J82sc) or TN-C over-expression (5637TN-CVs 5637Vec and 253JTN-CVs 253JVec), GAPDH was a loading control; E. Real time PCR indicates the efficiency of TN-C knockdown (T24si TN-CVs T24sc and J82si TN-C Vs J82sc) or TN-C over-expression (5637TN-CVs 5637Vec and 253JTN-CVs 253JVec), $*P < 0.05$; F. ELISA data depicting TN-C in medium from cells with TN-C knocked down or with TN-C stably overexpressed, $*P < 0.05$.

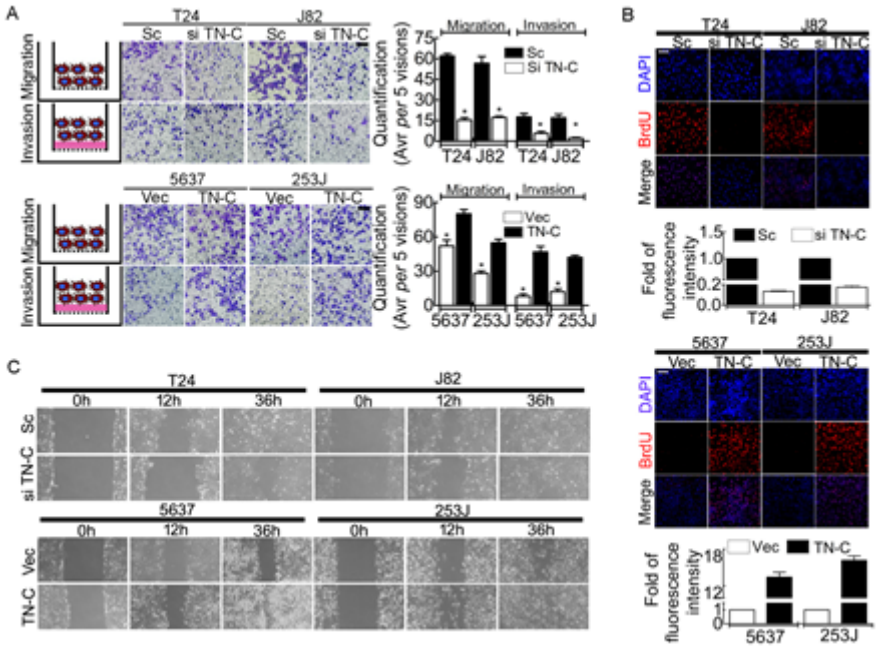

Figure 3

TN-C contributes to BCa cell line proliferation, migration and invasion A. Boyden chamber assay, indicating that TN-C contributes to tumor cell migration and invasion. Left: cartoon of tumor cell with or without Matrigel (pink area), middle: representative figures, Bar: 100 μm; right: quantification, *P < 0.05; B. BrdU incorporation, indicating that TN-C contributes to tumor cell proliferation. Upper: representative figures, bar: 100 μm; lower: relative fluorescent intensity; C. Wound healing analysis at 0, 12, and 35 h suggesting that TN-C leads reduces wound healing time, Bar:100 μm.

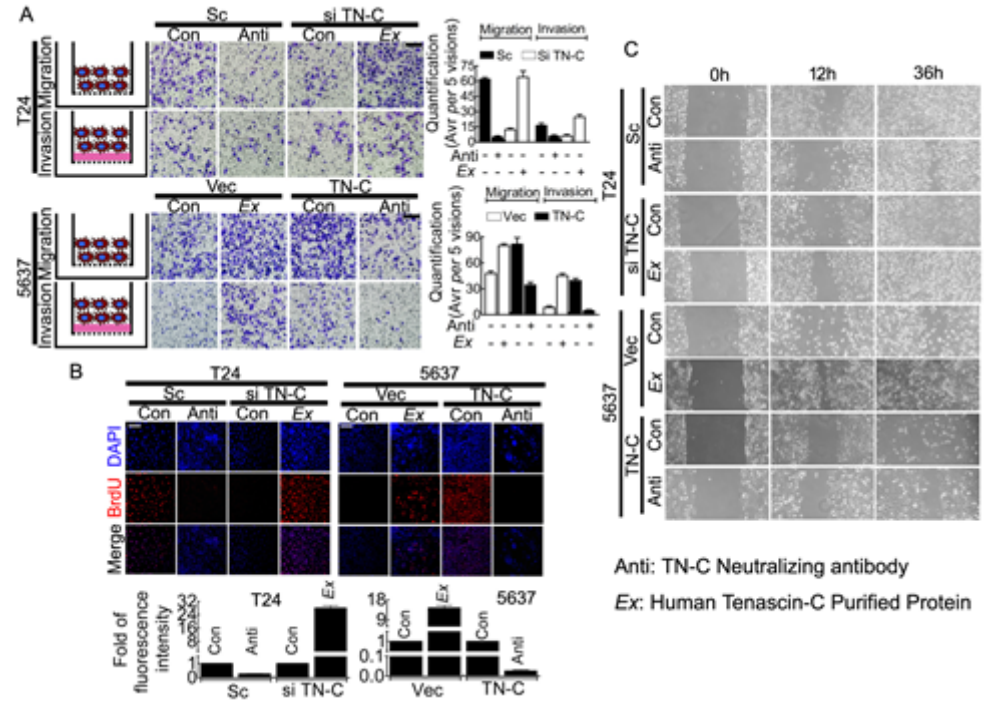

Figure 4

TN-C executes its roles as a secreted protein in BCa cell lines, at least partially A. Boyden chamber assay with Ex or Anti, left: representative figures, Bar:100 μm; right: quantification, *P < 0.05, indicating that exogenous TN-C promotes tumor cell migration and invasion (T24si TN-C-Ex Vs T24si TN-C-con, 5637Vec-Ex Vs 5637Vec-con), whereas the neutralizing antibody of TN-C attenuates this phenomenon (T24sc-Anti Vs T24sc-con, 5637TN-C-Anti Vs 5637TN-C-con); B. BrdU incorporation in the presence of Ex or Anti, upper: representative figures, Bar: 100 μm; lower: relative fluorescent intensity, suggesting that exogenous TN-C leads to tumor cell proliferation (T24si TN-C-Ex Vs T24si TN-C-con, 5637Vec-Ex Vs 5637Vec-con), and TN-C neutralizing antibody inhibits this (T24sc-Anti Vs T24sc-con, 5637TN-C-Anti Vs 5637TN-C-con); C. Wound healing analysis with Ex or Anti, indicating that exogenous TN-C reduces wound-healing time, whereas neutralizing antibody prolongs this duration, Bar: 100 μm.

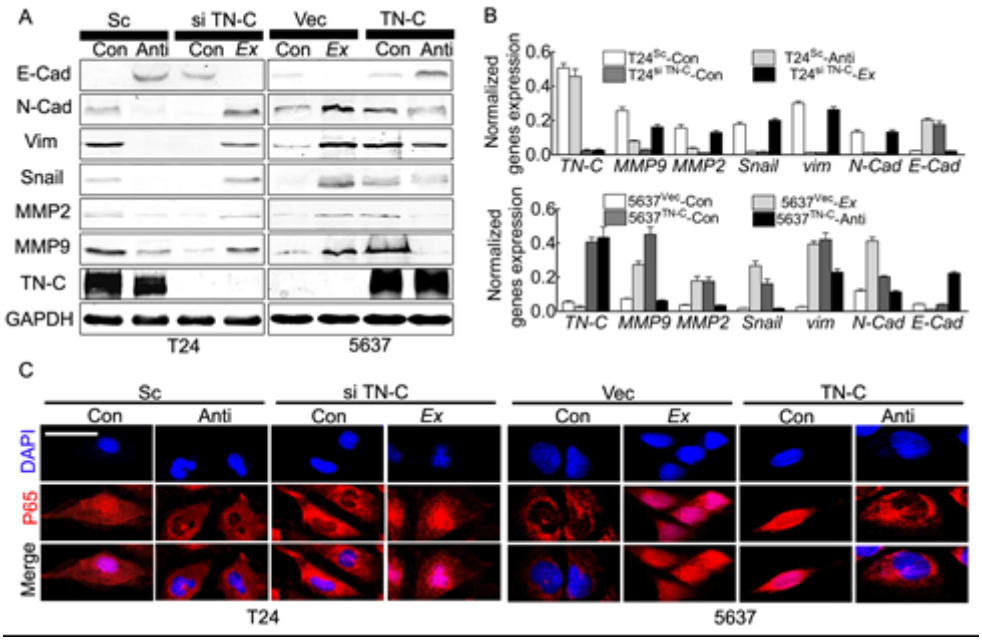

Figure 5

Secreted TN-C induces activation of NF-κB signaling, leading to EMT A. Western blot indicates that secreted TN-C induces upregulation of N-Cad, Vim, Snail, MMP2 and MMP9 and downregulation of E-Cad; GAPDH was a loading control; B. real time PCR agrees with Western blot; C. Immunofluorescent staining suggests that secreted TN-C contributes to nuclear translocation of NF-κB (P65), activating signaling, Bar: 20 μm;

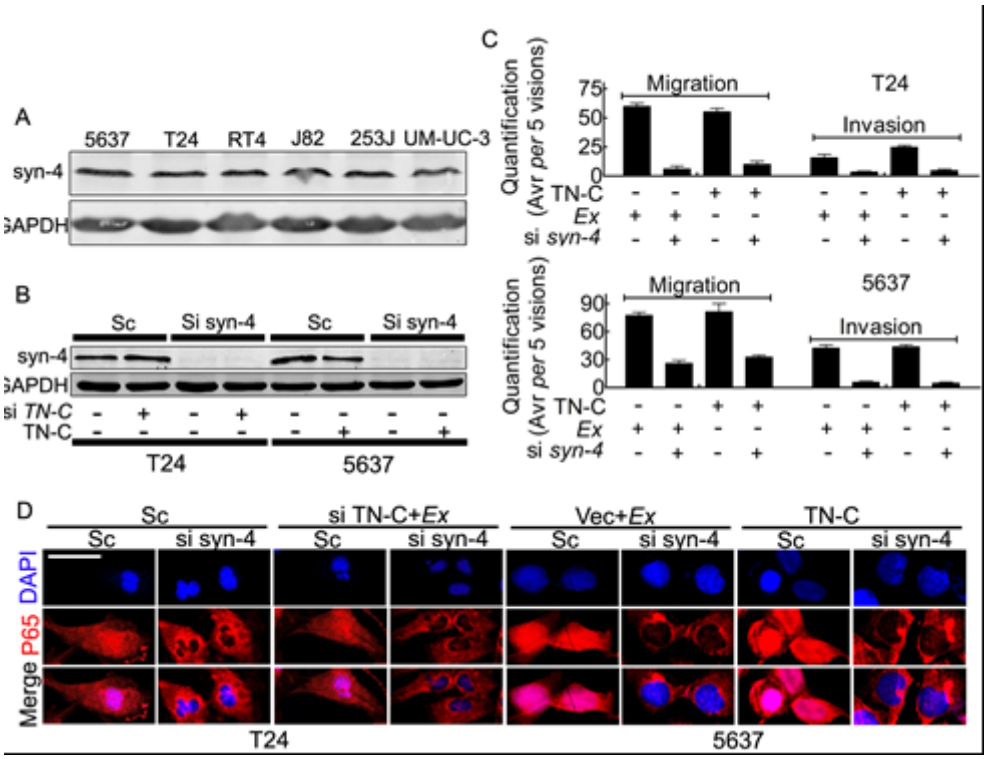

Figure 6

TN-C contributes to activation of NF-κB signaling depends on membrane receptor syndecan-4. A. Western blot of syndecan-4 (syn-4) expression in all BCa cell lines, indicating ubiquitous expression; GAPDH was a loading control; B. Western blot indicates no visible discrepancy of syn-4 expression in T24sc Vs T24siTN-C and 5637Vec Vs 5637TN-C, and siRNA to knock down syn-4 significantly attenuated expression of syn-4 in both cell lines, indicating the perfect efficiency; C. quantification of Boyden chamber assay indicates knocking down syn-4 in both cell lines decreases migration and invasion in the presence of TN-C, Ex: exogenous human TN-C; D. Immunofluorescent staining indicates that TN-C induced nuclear translocation of NF-κB is diminished without syn-4 but with TN-C, Bar: 20 μm;

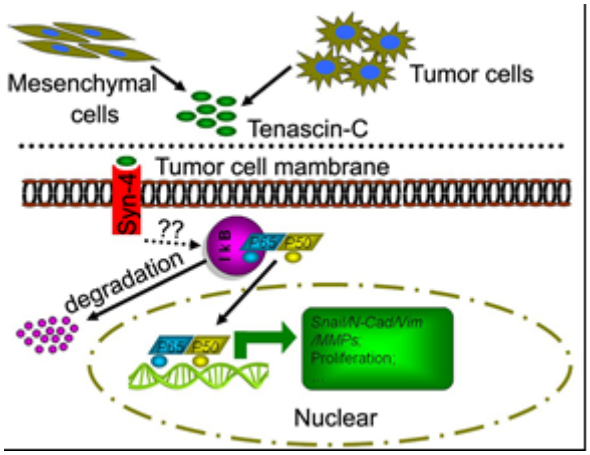

Figure 7

Data summary. In the tumor microenvironment TN-C may be secreted by diverse cells including tumor cells, fibroblasts and other mesenchymal cells. In tumor cells, secreted TN-C binds to the membrane receptor syndecan-4 and a critical downstream factor for this is NF-κB signaling. Binding of TN-C to syndecan-4 leads to activation of NF-κB signaling and subsequent enhanced ability of cells to migrate, invade, and proliferate as well as cause EMT. However, how of how syndecan-4 activates NF-κB signaling is not known.