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Original Article

Tenascin-C expression is associated with poor prognosis in hepatocellular carcinoma (HCC) patients and the inflammatory cytokine TNF-α-induced TNC expression promotes migration in HCC cells

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Abstract: Although tenascin-c (TNC) in inflammatory microenvironment contributes to progression in some tumors, its role in hepatocellular carcinoma (HCC) in metastasis and the mechanism by which TNC expression is regulated in HCC cells are elusive. In this study, we examined TNC expression in 100 HCC tissue samples by immunohistochemistry and compared which between the groups with or without metastasis. TNC expression was higher in metastatic HCC tissues than that in the non-metastatic HCC tissues, which was associated with the Knodell inflammation scores. Importantly, high level of TNC expression was associated with lower survival rate and shorter survival time in the HCC patients. We then investigated the mechanism by which TNC expression is regulated in HCC cells with an in vitro cell culture system. The recombinant TNF-α and conditioned medium from macrophages induced TNC expression at both mRNA and protein levels in HepG2 cells. The induction of TNC expression by conditioned medium from macrophages was suppressed by a TNF-α neutralizing antibody. TNF-α-promoted cell migration was inhibited by a TNC siRNA. In addition, TNF-α-induced TNC expression was blocked by a NF-κB pathway inhibitor. These results suggest that TNF-α in the tumor microenvironment induces TNC expression in HCC cells through the NF-κB pathway, which in turn, promotes HCC cell migration. Thus, TNC may play an important role in promoting HCC metastasis and TNC expression could be a predictive factor for poor prognosis in HCC patients.

Keywords: Tenascin-c, TNC, hepatocellular carcinoma, TNF-α, cell migration, metastasis

Introduction

Liver cancer is the third most common cause of cancer-related death, which has a low 5-year survival rate of 4.4~6% and high 5-year recurrence rate of 43.5~61.5% even after curative resection [1]. High metastasis rate is the main reason of therapy failure and high mortality rate in hepatocellular carcinoma (HCC) patients. Although a few molecular markers have been shown to have a prognosis potential in HCC [2], specific biomarkers that provide preventive or therapy targets for HCC metastasis have not been determined.

Tenascin-C (TNC) is a large hexameric extracellular matrix glycoprotein that is highly expressed in malignant solid tumors. While TNC expression is high in fetal tissues, it is restrictedly and lowly expressed in normal tissues [3, 4]. With various domains and complicate alternatively spliced isoforms, TNC has multiple functions in cell adhesion, tissue remodeling and tumor cell migration and invasion [5]. While it has been reported that TNC plays an important role in tumor progression in various cancers such as colorectal cancer, breast cancer, glioblastoma and glioma [6-9], the expression and function of TNC in HCC have not been well elucidated [10].

It has been reported that TNC expression is tightly associated with early inflammatory responses and up-regulation of TNC expression

Table 1. Clinicopathologic Characteristics of the HCC Patients

Characteristics	Metastasis HCC	Non-metastasis HCC	x^2 *value*	*P value*	Spearman's rho	*P value*
Age (y)						
≤ 50/> 50	32/18	19/31	6.673	0.009	0.260	0.009
Gender						
Female/Male	6/44	4/46	0.444	0.505	-0.067	0.510
ALT (IU/L)						
< 55/≥ 55	31/19	34/16	0.396	0.529	0.063	0.534
AST (IU/L)						
< 46/≥ 46	20/30	30/20	4.000	0.046	-0.200	0.046
HBsAg						
Positive/Negative	46/4	43/7	0.919	0.338	0.096	0.343
HBeAg						
Positive/Negative	8/38	9/34	0.180	0.671	-0.045	0.675
HBV DNA (copies/ml)						
< 1000/≥ 1000/not-detected	9/17/20	11/23/9	0.034	0.854	0.024	0.857
Ishak score						
6/5/4/3/2/1/not-evaluated	23/5/9/8/1/0/4	17/11/6/10/1/2/3	6.115	0.410	0.056	0.578
AFP (ng/ml)						
< 8/≥ 8	4/46	22/28	16.840	0.000	-0.410	0.000
Tumor size(cm)						
≤ 5/> 5	10/40	34/16	23.377	0.000	-0.483	0.000
Differentiation						
Low/Moderate/High	38/12/0	7/40/3	39.432	0.000	0.617	0.000
Necrosis						
Yes/No	27/23	6/44	19.946	0.000	0.447	0.000

HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; AST, aspartate transaminase; AFP, alpha fetoprotein. x^2 value and corresponding *P* values were calculated by Chi-Square test. Spearman's correlation analysis and *P* values were calculated by Log-rank test.

may contribute to tumor invasion [11, 12]. Inflammation is involved in tumor invasion and metastasis, which involves secretion of inflammatory cytokines, growth factors and proteases, and remodeling of the tumor microenvironment [13]. TNF-α, a common inflammatory cytokine that promotes inflammatory reaction in liver through activating the NF-κB pathway, possesses a pro-carcinogenesis activity in the liver [14, 15]. Persistent inflammatory reaction due to hepatitis virus infection is a major risk for liver cirrhosis and HCC [16, 17]. Because TNC expression is closely associated with inflammation, it is plausible to hypothesize that TNC contributes to HCC progression. However, solid evidence supporting this hypothesis is still lacking.

In the present study, we investigated the expression of TNC in HCC tissues and the role of TNC in HCC progression. The results showed that high level of TNC expression in HCC was associated with lower patient survival rate and shorter survival time. TNC expression was closely correlated to inflammation. Furthermore, we demonstrated that up-regulation of TNC expression by TNF-α facilitates the migration ability in HCC cells, which involves the NF-κB pathway. The results suggest that TNC plays an important role in promoting HCC metastasis and high level of TNC expression could be a predictive factor for poor prognosis in HCC patients.

Materials and methods

Patients

One hundred patients that diagnosed as HCC and underwent surgery between October 2010 and December 2012 at West China Hospital, Sichuan University, China were included and

HCC tissue samples were obtained. All patients were confirmed to have primary HCC by surgical excision biopsy. No patients had received irradiation or chemotherapy prior to surgery. Eleven normal liver tissue samples without any liver diseases from the West China Hospital, Sichuan University, China, were used as normal control. Informed consents were obtained from all the patients or their relatives prior to analysis, and the project was approved by the Ethics Committee of West China Hospital, Sichuan University.

The patients were allocated to metastatic and non-metastatic groups. The metastatic HCC group included 50 patients, was had capsule invasion, vascular thrombosis, portal vein thrombosis, intra-hepatic metastasis, lymphatic metastasis and extra-hepatic infringement [18-20]. The other 50 patients without the characteristics above were assigned to the non-metastatic HCC group. The clinicopathologic characteristics of subgroups were summarized respectively in **Table 1**.

Reagents

Anti-TNC rabbit polyclone antibody was purchased at Santa Cruz Biotechnology (CA, USA). Anti-β-actin was from Cell Signaling Technology (Danvers, MA, USA). The JNK inhibitor SP600125 and IKK inhibitor IKKin were from Calbiochem (La Jolla, CA, USA). Lipopolysaccharide (LPS), phorbol myristate acetate (PMA) and TNF-α were from Sigma (St. Louis, MO, USA). The TNF-α neutralizing antibody and negative control antibody were from Cell Signaling Technology (Danvers, MA, USA).

Tissue preparation and immunohistochemistry staining

Tissue samples were fixed in 10% formalin for 48 hours and embedded with paraffin. Histological analysis was performed on 4 μm tissue sections by staining with hematoxylin and eosin. TNC was detected by immunohistochemistry by using a specific anti-TNC antibody, which was incubated overnight at 4°C with a dilution of factor of 1:250.

The immunostaining results were independently observed and interpreted by two pathologists unaware of clinical data. The level of TNC immunoreactivity was expressed with stain grade, accumulated by the amount of TNC positive-stained cancer cells or the positive-stained mesenchymal area plus staining intensity. The percentage of positive cells and mesenchymal area was scored as follows: 0 = 0~10%; 1 = 11~25%; 2 = 26~50%; 3 = 51~75%; 4 = 76~100%. And the intensity of positive staining was scored as follows: 0 = no staining; 1 = low staining; 2 = moderate staining; 3 = strong staining. The Knodell score was used to evaluate the inflammatory status [21].

Cell culture and preparation of conditioned medium

Human hepatoma cell line HepG2 and human monocyte cell line THP-1 were grown in DMEM with 10% fetal bovine serum, 1 mM glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Conditioned medium (CM) were prepared by adding PMA (100 ng/ml) to the medium of THP-1 and cultured overnight. When the suspended THP-1 cells turned to adherent cells, they had already differentiated into macrophages. Then the cells were treated with LPS (10 ng/ml) up to 48 hours, the medium were collected and the concentration of TNF-α in the medium were measured by ELISA and preserved as conditioned medium at -70°C respectively for use.

Transwell invasion and migration assays

Transwell invasion and migration assays were performed by using transwell chamber (8 μm pore size) coated with matrigel (BD Biosciences, San Diego, CA) [22]. HepG2 cells were serum starved for 24 hours and plated in 12-well plates, then treated as indicated in each figure legend. Cells were collected and plated into transwell chambers with 5×10^4 cells/chamber. After incubating for 24 hours, cells were fixed by methanol for 30 mins and stained with 0.1% crystal violet for 20 mins. Cells were counted in random 20 fields after removing the remaining cells from the upper surface of the filter.

siRNA-mediated TNC knockdown

siRNAs targeting human TNC (GUGGAGAGCUUCCGGAUUATT) and negative control (UUCUCCGAACGUGUCACGUTT) was purchased from Dharmacon (Denver, CO, USA). HepG2 cells were transfected with TNC siRNA or negative

control-siRNA at a 5 nM final concentration by using INTERFERin™ (PolyPlus-Transfection, San Marcos, CA) according to manufacturer's instructions. After transfection for 24 hours, cells were treated with TNF-α. TNC expression was detected by reverse transcription-PCR and Western blot. Cell migration was detected by transwell assays.

Western blot

Cells were harvested and lysed in M2 buffer (20 mM Tris-HCl, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 μg/ml leupeptin). Equal amounts of protein extracts were resolved by 12% SDS-polyacrylamide gel electrophoresis, and the proteins were detected by Western blot and visualized by enhanced chemiluminescence according to the manufacturer's instructions (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

RT-PCR

Total RNA was extracted with an RNeasy Kit (QIAGEN, Valencia, CA). One microgram of RNA from each sample was used as a template for cDNA synthesis with a reverse transcription kit (Promega). An equal volume of cDNA product was used in the PCR. The primers used were: TNC, 5'-TGTAAAACGACGGCCAGT-3' and 5'-CAGGAAACAGCTATGACC-3'; and β-actin, 5'-CCAGCCTTCCTTCCTGGGCAT-3' and 5'-AGGAGCAATGATCTTGATCTTCATT-3'. The reaction condition was 94°C for 45 s, 55°C for 40 s, and 72°C for 45 s. For TNC and β-actin, the cycles for PCR were 27 and 21, respectively. PCR products were run on 1% agarose gels with 0.5 μg/ml ethidium bromide, visualized, and photographed.

Statistical analysis

Data were analyzed by using SPSS statistical software package, version 16.0 (SPSS Inc., Chicago, IL, USA). The immunostaining scores of TNC and Knodell score in HCC tissues were presented as the median (minimum, maximum) of each sample. The Chi-Square test and the Mann-Whitney U test were used to compare variables between two groups. Spearman's correlation coefficient was used to assess the strength of associations between variables. Survival analysis was performed by Kaplan-Meier survival curves. *P* values less than 0.05 were considered to be statistically significant.

Results

Clinicopathologic characteristics of the HCC patients

The clinicopathologic characteristics of HCC patients with or without metastasis are summarized in **Table 1**. Patients in the metastatic HCC group had a larger tumor size than that in the non-metastatic HCC group. Similarly, most HCC cases with metastasis showed poor differentiation, but the non-metastasis group tended to have a higher differentiation grade. Up to 54% of the patients in the metastasis group, while there were only 12% of the patients in non-metastasis group, had necrosis. Collectively, male, young age, large tumor size, low differentiation, high AFP level and necrosis were associated with metastasis. Furthermore, correlation analysis showed that there were statistic significances of these characteristics (except gender) between the metastatic and non-metastatic subgroups (**Table 1**).

Increased TNC expression in metastatic HCC is closely associated with higher inflammation and poor prognosis in HCC patients

TNC was mainly detected in tumor cell plasma. TNC was also detected in mesenchymal region including hepatic sinusoidal walls and stroma in both metastatic HCC and non-metastatic HCC groups (**Figure 1A**). In normal liver tissues, TNC was weakly detected in the sinusoidal walls (**Figure 1A**). The total TNC score (expression density and the distribution area of TNC) in the metastatic group was higher than that of the non-metastatic group (**Figure 1B**; scored 4.456 ± 0.2664 and 2.502 ± 0.1931, respectively; Supplemental Table 1). The total TNC scores of the metastatic and non-metastatic group are higher than that of the normal tissues (scored 0.588 ± 0.1043; **Figure 1B**). A similar TNC expression trend was found in cancer cell and in mesenchyme in both metastatic and non-metastatic groups, respectively (Supplemental Table 1). Thus, these results suggest that high TNC expression was associated with metastasis potential.

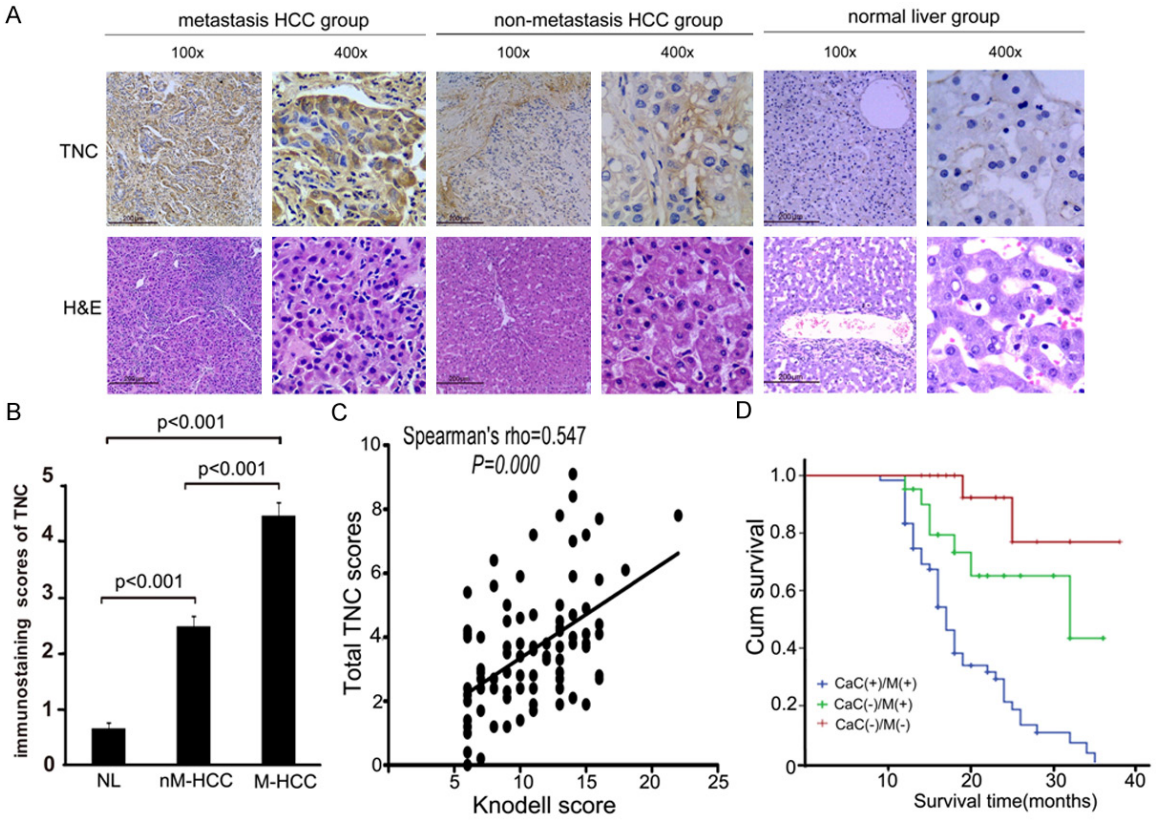

Figure 1. The association of TNC Expression and inflammation in HCC patients and Kaplan-Meier survival curves for all HCC patients with different TNC expression levels. A. TNC was detected by immunohistochemistry in normal liver tissues and in HCC tissues with or without metastasis. H&E staining in normal liver and in HCC tissues with or without metastasis tissues were used to evaluate the inflammatory status by Knodell score system. Bar: 100x, 200 μm. B. Statistic analysis TNC expression in HCC patients with or without metastasis and in normal liver tissues. *P* values calculated by Mann-Whitney U test. C. Total TNC scores and the corresponding Knodell scores were analyzed by Spearman's correlation. *P* values were calculated Log-rank test. D. Survival analysis was performed by Kaplan-Meier survival curves for all HCC patients with different TNC expression. H&E, hematoxylin and eosin; NL, normal liver; nM-HCC, non-metastasis HCC; M-HCC, metastasis HCC; CaC(+)/M(+), cancer cell positive/mesenchyme positive of TNC; CaC(-)/M(+), cancer cell negative/mesenchyme positive of TNC; CaC(-)/M(-), cancer cell negative/mesenchyme negative of TNC.

To determine the relationship between inflammation and TNC expression, Knodell score system was applied to assess the inflammation level. The results showed a higher inflammation score in metastatic HCC group compared to that in the non-metastatic group (12.72 ± 3.39 and 8.58 ± 2.52, respectively; Supplemental Table 1). Correlation analysis of total TNC scores was performed. There was a close association between total TNC scores and Knodell scores (**Figure 1C**, spearman's rho = 0.547, P < 0.01). Thus, TNC expression was associated with inflammation in the liver tissue in HCC patients.

A post-surgery follow-up was conducted to evaluate the relationship between patient survival and TNC expression. Patients were classified into three groups according to the deposition of TNC in cancer cell or in mesenchyme: cancer cell positive/mesenchyme positive (CaC+/M+, 60 patients), cancer cell positive/mesenchyme negative (CaC+/M-, 21 patients) and cancer cell negative/mesenchyme negative (CaC-/M-, 19 patients). During the follow-up, 48 patients in CaC+/M+ group, 7 patients in CaC+/M- group and 2 patients in CaC-/M- group have died. There was a significant difference among the three groups (χ^2 = 29.750, P < 0.05) (see Supplemental Table 2). Kaplan-Meier survival curves indicated that patients in CaC+/M+ group had the shortest median survival time (17 months). The patients in CaC+/M- group survived longer (32 months) (**Figure 1D**). These

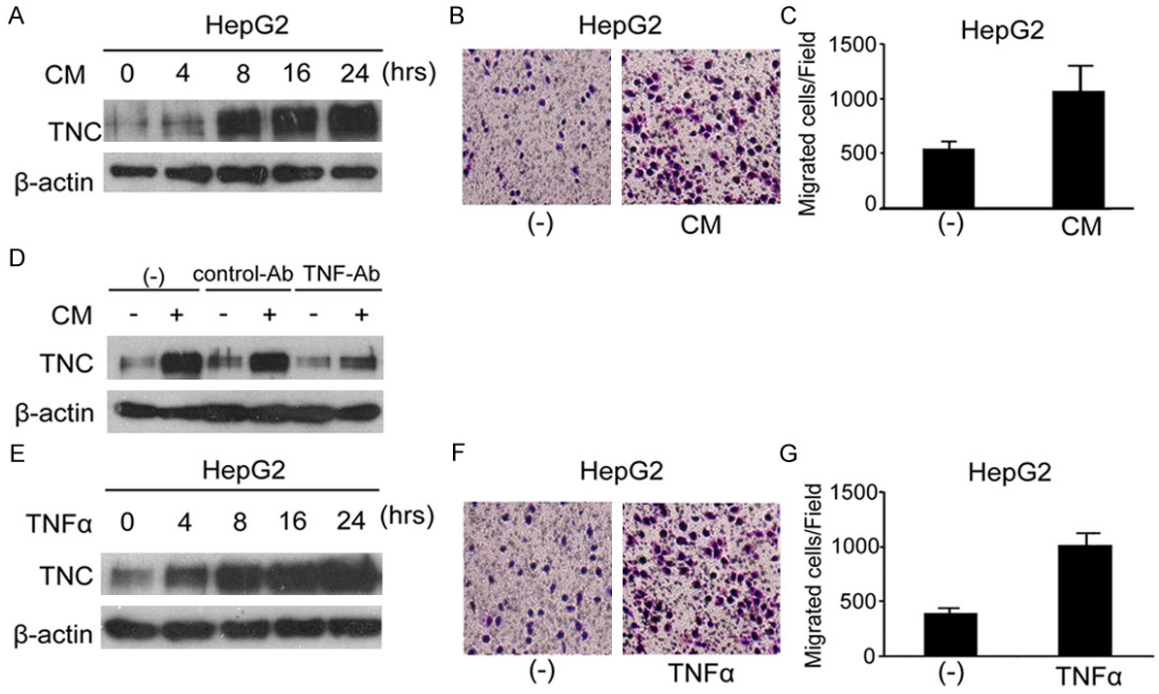

Figure 2. Inflammatory cytokines promoted HepG2 invasion and migration by up-regulating TNC expression. A. HepG2 cells were treated with conditioned medium (CM) from THP-1 for the indicated times or left untreated. TNC was detected by Western blot. B, C. HepG2 cells were pretreated with THP-1 CM for 16 h or left untreated and then re-suspended to a concentration of 5×10^5/ml. 100 ul of cell suspension were added to the upper transwell chamber and cultured for 48 hours. The cells that invaded through the matrigel and crossed the membrane were fixed, stained and counted in random 20 fields under 400 × magnification. D. HepG2 cells were pretreated with control-antibody (1 μg/ml) and TNF neutralizing antibody (1 μg/ml) for 1 hour or left untreated, followed by 16 hours of treatment with THP-1 CM or not. TNC was detected by Western blot. E. HepG2 cells were treated with TNF-α (10 ng/ml) for the indicated times or left untreated. TNC was detected by Western blot. F, G. HepG2 cells were pretreated with TNF-α (10 ng/ml) for 16 hours or left untreated. Transwell assays were performed as described in B and C.

results strongly suggest that patients with high TNC expression levels were closely associated with poor prognosis.

TNF-α promoted HepG2 migration by up-regulating TNC expression

Next, we employed the in vitro cell culture system to investigate the role of TNC in HCC cell migration and the mechanisms by which TNC expression in HCC cells is up-regulated by inflammatory cytokines. We hypothesized that TNC expression induced by inflammatory cytokines promotes HCC cell migration. The macrophage cell line THP-1 was used as a source of inflammatory cytokines. Conditioned medium (CM) from THP-1 cells stimulated by LPS potently promoted TNC expression in HepG2 cells, which started as early as 8 hours after treatment (**Figure 2A**). Consistently, the migration ability of HepG2 detected by transwell assay was increased by the THP-1 CM (**Figure 2B**, **2C**). Because TNF-α is the major component of the conditioned medium (data not shown), we hypothesized that TNF-α was the factor involved in induction of TNC expression and migration in HepG2 cells. Indeed, a TNF-α neutralizing antibody effectively attenuated the effect of THP-1 CM on TNC expression (**Figure 2D**). Furthermore, a recombinant TNF-α effectively promoted the TNC expression and migration in HepG2 cells (**Figure 2E-G**). These results imply that TNF-α is the effective factor in the HCC tumor microenvironment for TNC expression and HCC metastasis.

Knockdown of TNC expression decreased HepG2 cell migration

In order to confirm the role of TNC in HCC cell migration, a specific TNC siRNA was used to knockdown the expression of TNC in HepG2 cells. Effective TNC knockdown was confirmed by Western blot and RT-PCR (**Figure 3A**, **3B**).

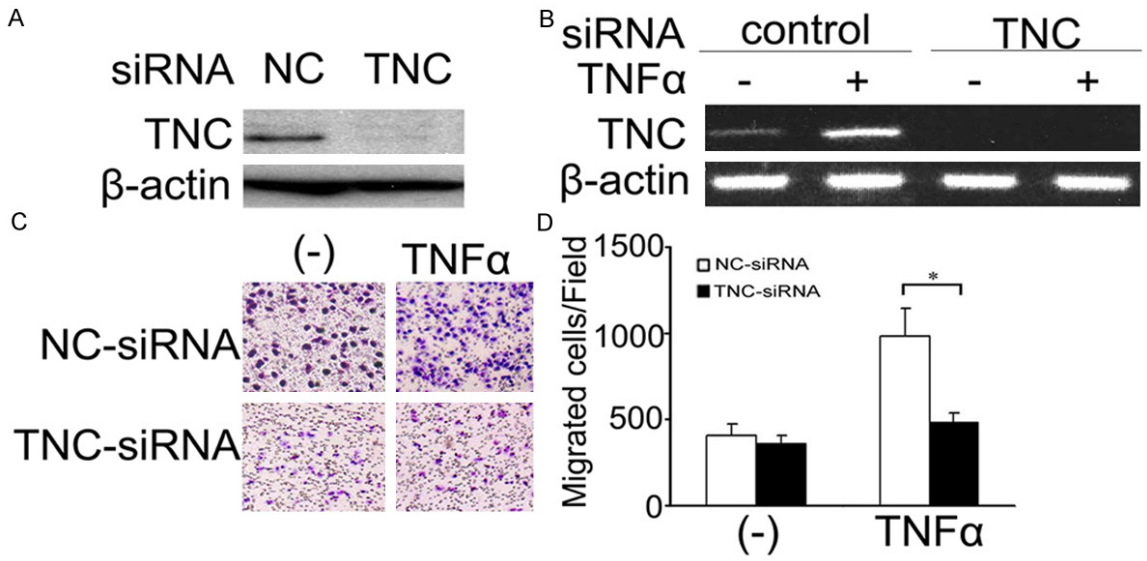

Figure 3. Interfering TNC expression attenuated the invasion and migration abilities of HepG2 cells. A. HepG2 cells were transfected with negative control siRNA (NC-siRNA) and TNCsiRNA (5 nM) for 48 hours. TNC was detected by Western blot. B. HepG2 cells were transfected with NC-siRNA and TNCsiRNA (5 nM) for 6 h or left untreated, followed by TNF-α treatment (10 ng/ml) for 2 hours or not. TNC mRNA was detected by reverse transcription-PCR. C, D. HepG2 cells were transfected with NC-siRNA and TNCsiRNA (5 nM) for 16 hours, followed by TNF-α treatment (10 ng/ml) for 16 hours or left untreated. Transwell assays were performed as described in **Figure 2B** and **2C**.

TNF-α-induced TNC expression was completely blocked by TNC siRNA (**Figure 3B**). TNC knockdown effectively suppressed TNF-α-promoted HepG2 cell migration ($p < 0.05$, **Figure 3C**, **3D**). These results strongly suggest TNC plays an important role in TNF-α-promoted HepG2 migration.

TNF-α Induces TNC expression through the NF-κB pathway

TNF-α exerts its function through cell signaling and JNK and NF-κB are major pathways activated by TNF-α [23]. Therefore, we investigated the role of JNK and NF-κB pathways in TNF-α-induced TNC expression with specific inhibitors, SP600125 for JNK and IKK inhibitor for NF-κB. The IKK inhibitor, but not SP600125, effectively blocked TNF-α-induced TNC expression and migration in HepG2 cells (**Figure 4**), suggesting that TNF-α up-regulated TNC expression in HepG2 cells through the NF-κB pathway for triggering cell migration.

Discussion

In this report, we examined the expression of TNC in HCC tissues with or without metastasis. Higher TNC expression levels in HCC tissues were associated with metastasis, and closely correlated to inflammation. We further confirmed that the inflammatory cytokine TNF-α promotes HepG2 cells migration through inducing the expression of TNC, in which the NF-κB pathways is pivotal. Our results highlight TNC in the inflammatory microenvironment as an important factor for HCC recurrence and metastasis.

TNC has been suggested to be a predictor or biomarker of tumor invasion and metastasis in numerous malignant cancers, and TNC has been investigated as a therapy target [24-27]. In this study, higher level of TNC expression was found in patients with metastatic HCC, and over-expression of TNC was closely correlated with poor prognosis in HCC patients. Although TNC has been studied in HCC by immunohistochemistry before, the distribution of TNC was not clear in HCC tissues except for one study that showed TNC was positively stained of tumor capsule [28, 29]. We found that TNC was mostly expressed in the perivascular districts, intracellular of tumor cell, as well as in the stroma adjacent to tumor nests. In contrast, in normal liver tissues, TNC was weakly detected as discontinuous staining in sinusoidal wall but not in hepatocytes, which was in lines with pre-

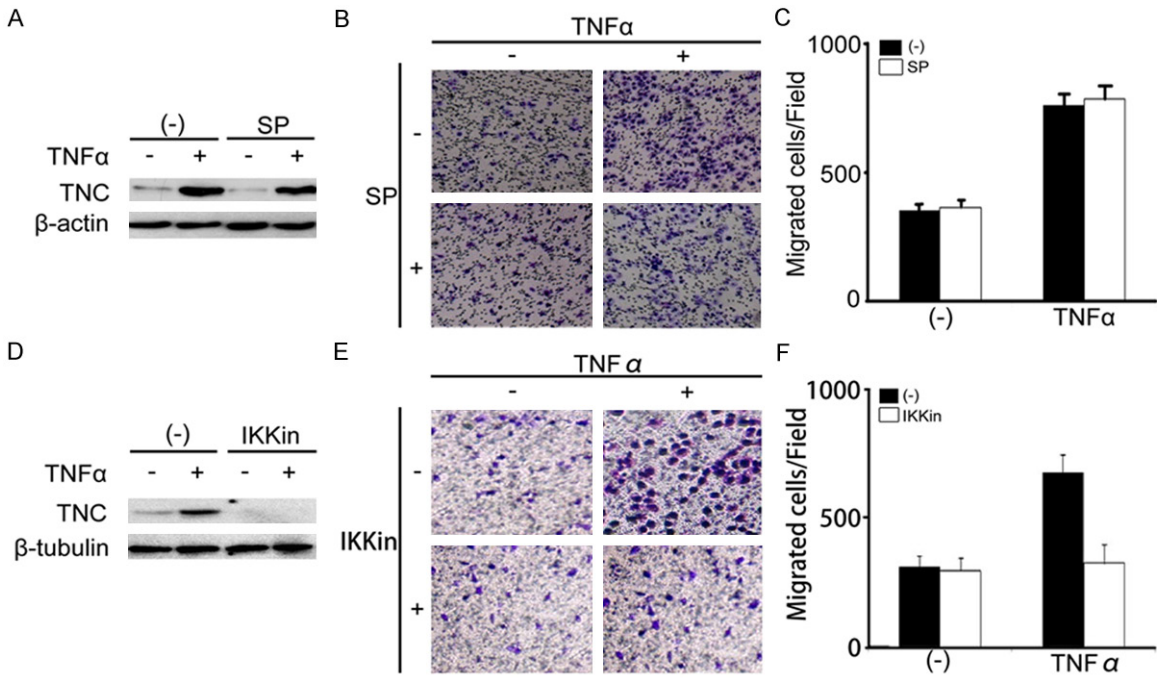

Figure 4. Blocking the NF-κB pathway inhibited TNF-α-induced TNC up-regulation and migration in HepG2 cells. A. HepG2 cells were pretreated with JNK inhibitor SP600125 (10uM) for 1 hour or left untreated and followed by TNF-α treatment (10 ng/ml) for 48 hours or not. TNC was detected by Western blot. B, C. HepG2 cells were pretreated with SP600125 (10 uM) for 1 hour or left untreated, followed by TNF-α treatment (10 ng/ml) for 16 hours or not. Transwell assays were performed as described in **Figure 2B** and **2C**. D. HepG2 cells were pretreated with NF-κB inhibitor IKKin (10 uM) for 1 hour or left untreated and followed by TNF-α treatment (10 ng/ml) for 48 hours or not. TNC was detected by Western blot. E, F. HepG2 cells were pretreated with IKKin (10 uM) for 1 hour or left untreated, followed by TNF-α treatment (10 ng/ml) for 16 hours or not. Transwell assays were performed as described in **Figure 2B** and **2C**.

vious studies [28, 30]. Our results provide new evidence supporting TNC as a biomarker for HCC recurrence and metastasis, and potential target for HCC therapy.

Several reports showed that TNC was increased in chronic hepatitis and cirrhosis tissues, and TNC was detected at the interface between parenchyma and mesenchyme, especially in the region of piecemeal necrosis [28, 30]. Therefore, low level of TNC around sinusoidal might be the inherent expression, and a transient or persistent up-regulation of TNC expression in this region may be resulted from temporary or persistent inflammation. Furthermore, it was suggested that inflammation is strongly associated with tumor progression. Thus, the inflammatory reaction in HCC tissue may be at least partly responsible for TNC expression in the mesenchyme. In this study, TNC expression was found to be expressed in both tumor and mesenchyme cells, and closely associated with worse prognosis in patients. However, when TNC was only expressed in mesenchyme, patients had better survival rates and lived longer.

Our results further suggest that inflammatory cytokines such as TNF-α derived from the inflammatory microenvironment facilitate migration of HCC cells, in which TNC plays an important role. We found that TNF-α up-regulates TNC expression in HCC cells to promote tumor cell migration. When TNC was suppressed with a specific siRNA, the migration of HepG2 was inhibited, suggesting that TNC is important for migration of HCC cells. A recent report has demonstrated that TNC can directly interfere with syndecan-4 binding to fibronectin, thereby inhibiting integrin-mediated signaling and further blocking cell adhesion to stimulate tumor cell proliferation [8]. Therefore, TNC might be the key factor that directly regulates migration of HCC cells and TNC may serve as a mediator for TNF-α-induced HCC cell migration and metastasis. It has been reported that JNK and NF-κB signaling pathways activated by TNF-α were associated with HCC progression

[14, 15]. Here we found that NF-κB, but not JNK, is involved in TNF-α-induced TNC expression. Thus, TNC appears to be one of the main NF-κB-regulated factors that are involved in TNF-α-induced HCC cell migration.

In conclusion, our results found that TNC is highly expressed in metastatic HCC tissues. The pro-inflammatory cytokine TNF-α induces TNC expression in HCC wells through the NF-κB pathway, which in turn, promotes HCC cell migration. The results suggest that TNC plays an important role in promoting HCC metastasis. Therefore, TNC could be a biomarker for HCC recurrence and metastasis, and potential target for HCC therapy.

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Disclosure of conflict of interest

None to disclose.

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Supplementary Table 1. Comparison of TNC immunohistochemistry score and knodell score between metastatic and non-metastatic HCC subgroups

Covariates	Metastatic HCC median (minimum, maximum)	Non-metastatic HCC median (minimum, maximum)	Z value	*P* value
Total TNC scores	4.1 (0.7, 9.1)	2.4 (0.0, 6.2.)	-8.647	0.000
Cancer cell TNC	1.8 (0.2, 6.6)	0.4 (0.0, 3.4)	-5.917	0.000
Mesenchyme TNC	2.0 (0.5, 3.0)	1.5 (0.0, 3.0)	-2.807	0.005
Knodell scores	14 (6.0, 22.0)	7.5 (6.0, 13.0)	-19.500	0.000

Z values and corresponding *P* values were calculated by using Mann-Whitney *U* test.

Supplementary Table 2. The relationship of different expression of TNC and the prognosis in patients with HCC

TNC expression	Survival	Dead	x^2 value	*P* value
CaC (+)/M (+)	12	48	29.750	< 0.05
CaC (-)/M (+)	14	7		
CaC (-)/M (-)	17	2		

CaC (+)/M (+), cancer cell positive/mesenchyme positive of TNC; CaC (-)/M (+), cancer cell positive/mesenchyme negative of TNC; CaC (-)/M (-), cancer cell negative/mesenchyme negative of TNC. x^2 value and corresponding *P* values were calculated by using the Chi-Square test.