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ORIGINAL ARTICLE

Neurotrophin 3 hinders the growth and metastasis of hepatocellular carcinoma cells*

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Abstract

Objective Neurotrophin 3 (*NTF3*) is involved in numerous biological processes; however, its role in hepatocellular carcinoma (HCC) is not well studied. This study investigated *NTF3* function in HCC progression and revealed its underlying molecular mechanisms.

Methods The prognostic relevance of *NTF3* was determined through a bioinformatical analysis of publicly available TCGA data. Immunohistochemistry of HCC biopsies was performed to explore the expression of *NTF3*. Cell growth and proliferation were analyzed using a Cell Counting Kit-8 (CCK-8) assay. Cell invasion and migration were analyzed using Boyden Transwell and wound healing assays. Protein expression and mRNA levels were evaluated through immunoblotting and quantitative polymerase chain reaction (qPCR). Cell apoptosis was evaluated with flow cytometry.

Results *NTF3* expression was significantly lower in HCC tissues than in adjacent non-tumor tissues. Low *NTF3* expression was significantly associated with decreased patient survival and specific clinicopathological features. *NTF3* overexpression reduced the proliferation, migration, and invasion abilities of HCC cell lines.

Conclusion Decreased expression of *NTF3* is associated with poor prognosis in HCC patients, likely due to its action in promoting HCC cell proliferation, migration, and invasion. Our findings provide a novel understanding into the pathogenesis of HCC and the role of *NTF3* in tumor progression, suggesting that targeting *NTF3* has potential therapeutic and diagnostic value for HCC.

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Hepatocellular carcinoma (HCC) is a highly lethal cancer with a rapidly increasing worldwide incidence. HCC accounts for 75%–85% of primary liver cancer cases [1–5]. Surgical treatment is one of the main forms of treatment for liver cancer, but chemotherapy, targeted therapy and immunotherapy will likely be recommended in the near future as non-invasive approaches [6–9]. However, early biomarkers and tumor-specific treatments for HCC are limited. A deeper understanding of the pathogenesis of HCC will be instrumental for early detection and treatment of the disease [10–11], which is why it is so important to find early diagnostic markers and novel therapeutic targets.

Neurotrophin-3 (*NTF3*) is a member of the neurotrophin family that includes nerve growth factor, brain-derived neurotrophic factors, and neurotrophin 4/5. *NTF3* is a growth factor that is involved in stem cell differentiation into neuron-like cells [12]. Previous studies of *NTF3* have focused on neuronal differentiation, osteoarthritic cartilage, neurogenesis, neural survival and Alzheimer's disease (AD) [12–18]. Research on *NTF3* in the field of cancer is rare and is limited to its role in breast cancer [19]. Previous studies have shown that in triple-negative breast cancer, *NTF3* is capable of activating TrkB to induce anoikis resistance [19].

Interestingly, our bioinformatics analysis indicated that *NTF3* may be involved in the development of HCC as a tumor suppressor gene. HCC has a very complex

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molecular pathogenesis and the underlying molecular mechanisms that initiate HCC involve several critical signaling pathways that promote the carcinogenic process [20–21]. There is evidence of enhanced anoikis-suppression through activation of the PI3K/Akt/Bcl-2 pathway in HCC cells [22]. Thus, we speculated that *NTF3* may exert an anti-tumor effect by inducing apoptosis of cancer cells.

This study aimed to explore the role of *NTF3* in the development of HCC and how it regulates this process. This study provides new insights into the molecular mechanisms underlying HCC progression and provides a new therapeutic target for HCC.

Materials and methods

Samples and informed consent

In total, 80 pairs of HCC and corresponding adjacent tissues (from areas in the vicinity (< 2 cm) of the tumor tissue with distinctly different edges) were obtained during surgical resections of patients without preoperative treatment at The Affiliated Hospital of Qingdao University (Qingdao, China). Human specimen collection was conducted in accordance with the guidelines of the Medical Ethics Committee of Affiliated Hospital of Qingdao University and approved by the Affiliated Hospital of Qingdao University Joint Institutional Review Board. All donors provided informed written consent prior to specimen collection according to the policies of the committee. The resected samples were identified by two pathologists independently.

Cell culture

In this study, one healthy liver cell line (HL-7702) and four HCC cell lines (SMMC-7721, Huh-7, BEL-7402 and HCCLM9) were used. All cell lines were obtained from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and cells were authenticated, tested for mycoplasma infection, and cultured in a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's Modified Eagle medium (DMEM) medium containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin and streptomycin. The medium was replaced every 2 days. Cells were monitored using microscopy to ensure that they maintained their original morphology.

Plasmid transfection

NTF3 overexpression plasmid constructs, *NTF3* shRNA, and their corresponding controls were provided by GeneChem. The plasmid overexpressing *NTF3* was transfected into SMMC-7721 cells, while the RNA-interference-treated *NTF3* plasmid was transfected into Huh7 cells. Endogenous *NTF3* expression was detected using real-time PCR after 24 h.

For the transfection experiments, cells were seeded in 24-well plates and incubated overnight at 37 °C with 5% CO_2. The above plasmids were transfected into the cells using Lip3000 (Life Technologies) according to the manufacturer's protocol. The HCC cells were then cultured for 24 h at 37 °C with 5% CO_2.

Wound-healing assays

For wound healing assays, cells were seeded into a 6-well plate and cultured at 37 °C for 24 h. Wounds were created in monolayers of cells using a 200 μL pipette tip. Cells were washed to remove cellular debris and incubated in DMEM without FBS at 37 °C with 5% CO_2. Images were taken at 0 h and 24 h after wounding. The wound area was measured and the percentage of wound healing was calculated using Image J software (NIH, Bethesda, MD, USA). This experiment was repeated three times.

CCK-8 assays

Cell proliferation was measured using the CCK-8 (Dojindo, Kumamoto, Japan). Briefly, the cell density of the treated cells was adjusted to 5×10^4 cells/mL with DMEM. Cells were then inoculated in a 96-well plate with 100 μL of cell suspension per well and incubated at 37 °C overnight. After culturing, the cells were washed and 10 μL of CCK-8 solution was added into each well of the plate. Cells were subsequently incubated for 4 h at 37 °C with 5% CO_2, and the absorbance was measured at 450 nm with a microplate spectrophotometer.

Transwell invasion assays

Cell invasion was measured using Matrigel-coated Transwell cell culture chambers. Cells in the logarithmic phase were starved in serum-free medium for 24 h, after which they were digested using 0.25% EDTA-trypsin. The cell suspension was then treated with serum-free medium, during which the density of the suspension was adjusted to 2×10^5/mL. Then, 100 μL of Matrigel with a final concentration of 1 mg/ml was added to the bottom of the upper chamber followed by incubation for 4–5 h at 37 °C to make it gelatinous. After the Matrigel was gelatinized, wells in the Transwell chamber were connected. The cell suspension was cultured in a 37 °C, 5% CO_2 incubator for 24 h. Three duplicate systems were used for each group. After 24 h, the chamber was dislodged, carefully cleansed once with PBS, and the cells were fixed with 70% ethanol for 1 h, then dried at room temperature. The chamber was then dyed with 0.5% crystal violet for 20 min, washed with PBS, and the upper side of the chamber was cleansed with a clean cotton ball. The migrated cells were wiped and cleaned, and the chamber was placed under an inverted microscope so that the remaining cells could be counted. The images were

analyzed using ImageJ software.

Apoptosis analysis

A total of 1×10^6 cells were cultured overnight and collected by trypsin digestion. The cells were washed with PBS followed by subsequent incubation at room temperature in the dark for 15 min, according to the manufacturer's protocol (AnnexinV-APC/7-AAD). Cell apoptosis was detected using a flow cytometer (BD Biosciences, USA).

Quantitative reverse transcription-PCR

Total RNA from tissues was extracted using Trizol (Takara) according to the instructions provided by the manufacturer and was treated with recombinant DNase I (RNase-free) (Code No. 2270A). Removal of genomic DNA was performed using gDNA Eraser. Reverse transcription was performed with 1 μg of RNA using RT Primer Mix mixed with Random 6 mers and Oligo dT Primer. Quantitative RT-PCR was performed using TB Green Premix (Takara, Otsu, Japan) in a LightCycler® 96 SW 1.1 machine (Roche). All reactions were performed in triplicate and GAPDH was used as internal control. The data were analyzed using the delta Ct method. Specific primer sequences for qRT-PCR were: NTF3-F (Forward): ATGATAAACACTGGAACTCT, NTF3-R (reverse): TAT CCGTATCCACCGCCAGC; GAPDH-F: TCATGGGTGT GAACCATGAGAA, GAPDH-R: GGCATGGACTGTGGT-CATGAG.

Western blot

Cells were scraped into RIPA buffer containing protease and phosphatase inhibitors. Extracted proteins were separated in 10% SDS-PAGE gels (Bio-Rad, 4561095) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked in 5% skim milk powder in Tris-buffered saline (TBS) with Tween 20 (TBS-T) for 2 h at room temperature. Incubation with primary antibodies was performed at 4 °C overnight. Membranes were washed with TBST, incubated with peroxidase-conjugated secondary antibody for 2 h and developed using the Enhanced Chemiluminescence (ECL) Detection System (Thermo Scientific). Antibodies were as follows: GAPDH (Cell Signaling #5174S, 1:1000), *NTF3* (abcam #Ab53685, 1:500), Caspase 3 (Cell Signaling #14220S, 1:1000), Bax (abcam #Ab32503, 1:1000), Bcl2 (Cell Signaling #3498S, 1:1000).

Statistical analysis

Statistical analysis was performed using the SPSS program (version 18.0; SPSS, Chicago, IL, USA). Data are presented as mean ± SD. Statistical significance was calculated using Student's *t*-test, χ^2 test, Fisher's exact test or one-way ANOVA. Pearson's analysis was used in correlation analyses. $P < 0.05$ was considered as statistically significant.

Results

NTF3 expression is decreased in HCC tissues and correlates with clinico-pathological characteristics

To identify genes with crucial roles in liver tumorigenesis, we first analyzed publicly available gene expression data from The Cancer Genome Atlas (TCGA), and screened genes that were differentially expressed in HCC tissues compared to normal tissues. As shown in Fig. 1a, several genes were identified with decrease or increased expression in HCC. *NTF3* was identified among the downregulated genes as having a potential association with liver tumorigenesis. A boxplot showing the expression of *NTF3* in HCC tissue compared with normal tissue is depicted in Fig. 1b. Survival analysis from the TCGA database indicated that low expression of *NTF3* was associated with poor survival of HCC patients while high expression of *NTF3* was associated with prolonged patient survival (Fig. 1c).

To experimentally verify the *in-silico* data, we collected clinical samples and assessed *NTF3* mRNA levels in HCC tissues and ANLTs by qRT-PCR. Consistent with the bioinformatical analysis results, the expression level of *NTF3* in HCC tissues was reduced compared to ANLTs (Fig. 2a). Immunohistochemistry was also performed on tumors and adjacent tissues collected from patients. Representative images of *NTF3* staining in the HCC and paracancerous tissue samples are shown in Fig. 2b. Negative *NTF3* staining was observed in HCC tissues, while normal adjacent tissues were positive for *NTF3* expression. Together, these results indicate that *NTF3* mRNA and protein expression is decreased in HCC tissues *in vivo*.

To explore the correlation between *NTF3* and clinicopathological variables, HCC tissues were divided into two groups: high and low *NTF3*-expression groups. Then, *NTF3* expression was correlated with clinicopathologic characteristics of HCC. The results (Table 1) indicated that gender, age, AFP and HBsAg were not associated with *NTF3* expression levels. In contrast, tumor size, the number of tumors present, tumor differentiation level, chronic hepatitis, liver cirrhosis, vascular invasion, invasion of nerves, TNM stage and BCLC were all inversely associated with *NTF3* expression. These observations indicate that decreased expression of *NTF3* is associated with tumor progression.

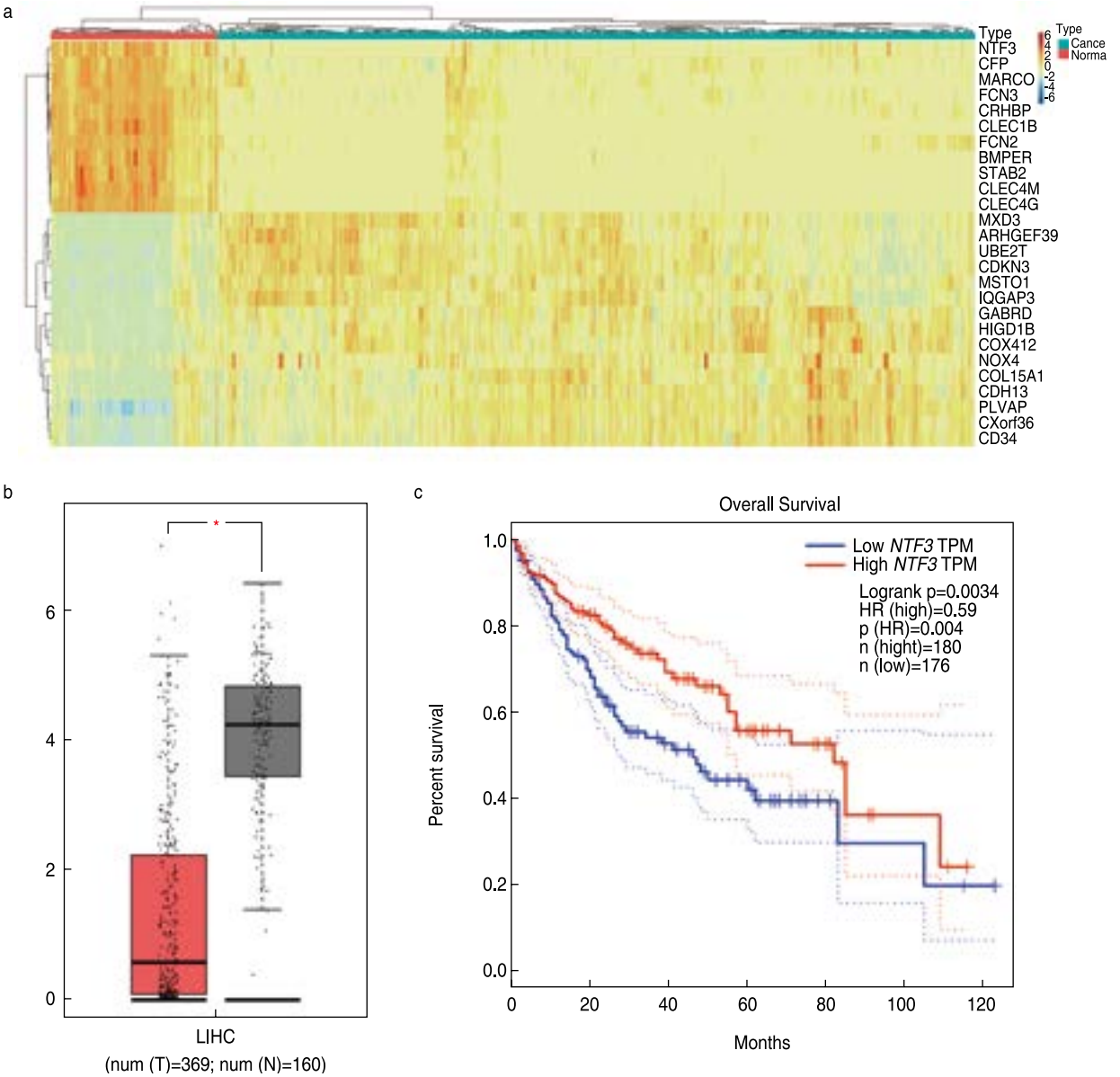

Fig. 1 Analysis of hepatocellular carcinoma (TCGA-LIHC). (a) Heatmap showing differentially expressed genes in HCC tumors and healthy samples; (b) Box plot showing the expression of *NTF3* in HCC tumors and healthy samples; (c) Effect of *NTF3* expression on the survival of LIHC patients

Expression of NTF3 in vitro and transfection efficiency analysis.

To explore the biological function of *NTF3*, *in vitro* experiments were performed using HCC cell lines. First, we measured the mRNA expression levels of *NTF3* in four HCC cell lines and healthy human hepatocytes using qRT-PCR. The results (Fig. 2c) indicated that *NTF3* expression was significantly decreased in the four HCC cell lines (Huh7, SMMC-7721, HCCLM9 and BEL-7402) compared to the healthy hepatocytes HL-7702. Among the four HCC cell lines, SMMC-7721 cells had the lowest average *NTF3* mRNA expression while Huh7 cells had the highest expression level. Thus, for functional analysis, *NTF3* was overexpressed in SMMC-7721 cells through cell transfection and silenced in Huh7 cells using *NTF3* shRNAs. The expression efficiency after shRNA silencing and overexpression are shown in Figure 2D and 2E, respectively. Transfection with the overexpression plasmid effectively and significantly increased the expression of *NTF3*, while transfection with three shRNAs markedly decreased *NTF3* expression.

Table 1 Correlation between the clinicopathologic chatacteristics and NTF3 expression in HCC tissues [*n* (%)]

Characteristics	Total number of patients (*n* = 80)	No. of patients		*P* value
		$NTF3^{low}$ *n* = 63	$NTF3^{high}$ *n* = 17	
Gender				
Male	66 (82.5)	52 (82.5)	14 (82.4)	1.0[b]
Female	14 (17.5)	11 (17.5)	3 (17.6)	
Age (years)				
≤ 60	49 (61.3)	37 (58.7)	12 (70.6)	0.79[a]
> 60	31 (38.8)	26 (41.3)	5 (29.4)	
Tumor size(cm)				
≤ 3	30 (37.5)	17 (27.0)	13 (76.5)	< 0.001†[a]
> 3	50 (62.5)	46 (73.0)	4 (23.5)	
Number of tumors				
1	38 (47.5)	25 (39.7)	13 (76.5)	0.0070†[a]
≥ 2	42 (52.5)	38 (60.3)	4 (23.5)	
Tumor differentiation level				
I–II	22 (27.8)	10 (15.9)	12 (70.6)	< 0.001†[b]
III–IV	57 (72.2)	53 (84.1)	5 (29.4)	
AFP (ng/mL)				
≤ 20	42 (52.5)	30 (47.6)	12 (70.6)	0.092[a]
> 20	38 (47.5)	33 (52.4)	5 (29.4)	
Chronic hepatitis				
No	21 (26.2)	8 (12.7)	13 (76.5)	< 0.001†[a]
Yes	59 (73.8)	55 (87.3)	4 (23.5)	
HBsAg				
Absent	46 (57.5)	38 (60.3)	8 (47.1)	0.33[a]
Present	34 (42.5)	25 (39.7)	9 (52.9)	
Liver cirrhosis				
Absent	21 (26.2)	10 (15.9)	11 (64.7)	< 0.001†[b]
Present	59 (73.8)	53 (84.1)	6 (35.3)	
Vascular invasion				
No	18 (22.5)	3 (4.8)	15 (88.2)	< 0.001†[b]
Yes	62 (77.5)	60 (95.2)	2 (11.8)	
Invasion of nerves				
No	39 (48.8)	22 (34.9)	17 (100.0)	< 0.001†[b]
Yes	41 (51.2)	41 (65.1)	0 (0.0)	
TNM				
I	15 (18.8)	4 (6.3)	11 (64.7)	< 0.001†[b]
I–III	65 (81.2)	59 (93.7)	6 (35.3)	< 0.001†[b]
IV	0 (0.0)	0 (0.0)	0 (0.0)	
BCLC				
0–A	30 (37.5)	20 (31.7)	10 (58.8)	0.041†[a]
B–C	11 (13.8)	6 (9.5)	5 (29.4)	0.0496†[b]
D	39 (48.8)	37 (58.7)	2 (11.8)	< 0.001†[a]
Child level				
A	19 (23.8)	5 (7.9)	14 (82.4)	< 0.001†[b]
B	22 (27.5)	20 (31.7)	2 (11.8)	0.13[b]
C	39 (48.8)	38 (60.3)	1 (5.9)	< 0.001†[a]

[a] Peason chi-squared test; [b] Fischer's exact test; † Bold text indicates statistical significance ($P < 0.05$)

NTF3 hinders HCC cell proliferation and promotes apoptosis in vitro

Cell Counting Kit-8 (CCK-8) assays were performed to assess the role of *NTF3* in the proliferation of HCC cells. Compared with the untransfected group and the negative control (NC) group, overexpression of *NTF3* in SMMC-7721 cells significantly decreased cell viability (Fig. 3a). In contrast, the rate of apoptosis in SMMC-7721 cells was markedly increased following *NTF3* overexpression (Fig. 3b and 3c). In addition, we found that silencing *NTF3* in Huh7 cells significantly promoted cell proliferation compared to the untransfected and NC groups (Fig. 3d). Moreover, flow cytometry analysis of cell apoptosis indicated that the rate of apoptosis in Huh7 cells was decreased after *NTF3* silencing (Fig. 3e and 3f).

To further confirm the effect of *NTF3* on cell apoptosis, Western blot analysis was performed on SMMC-7721 cells overexpressing *NTF3* and Huh7 cells in which *NTF3* was silenced. *NTF3* overexpression significantly increased the expression of cleaved-caspase 3 and Bax but decreased the expression of Bcl2 in SMMC-7721 cells (Fig. 4a, 4b, 4d, and 4e). Furthermore, silencing of *NTF3* caused a decrease in the expression of cleaved-caspase 3 and Bax, but increased the expression of Bcl2 in Huh7 cells (Fig. 4a, 4c, 4d, and 4f). These results indicate that *NTF3* hinders HCC cell proliferation and induces apoptosis *in vitro*.

NTF3 hinders HCC cell migration and invasion in vitro

Transwell and wound healing assays were carried out to explore the effects of *NTF3* on HCC cell migration. The Transwell assay indicated that overexpression of *NTF3* could inhibit the invasive activity of SMMC-7721 cells while knockdown of *NTF3* could promote the invasive activity of Huh7 cells (Fig. 5a and 5c). The wound healing assay showed that the wound closure of SMMC-7721 cells overexpressing *NTF3* proceeded slower than that of the untransfected and NC groups (Fig. 5b), whereas suppression of *NTF3* expression in Huh7 cells resulted in faster wound closure compared to the two control groups (Fig. 5d). These data suggest that *NTF3* can inhibit the metastasis of HCC cells *in vitro*.

Discussion

Regardless of the efforts made in anti-cancer research, patients with HCC still have a poor prognosis [23–26]. To uncover effective biomarkers for improving the diagnosis and prognosis of HCC, we examined the functions of *NTF3* in HCC *in vivo* and *in vitro*. We determined the expression level and the role of *NTF3* in HCC cells using

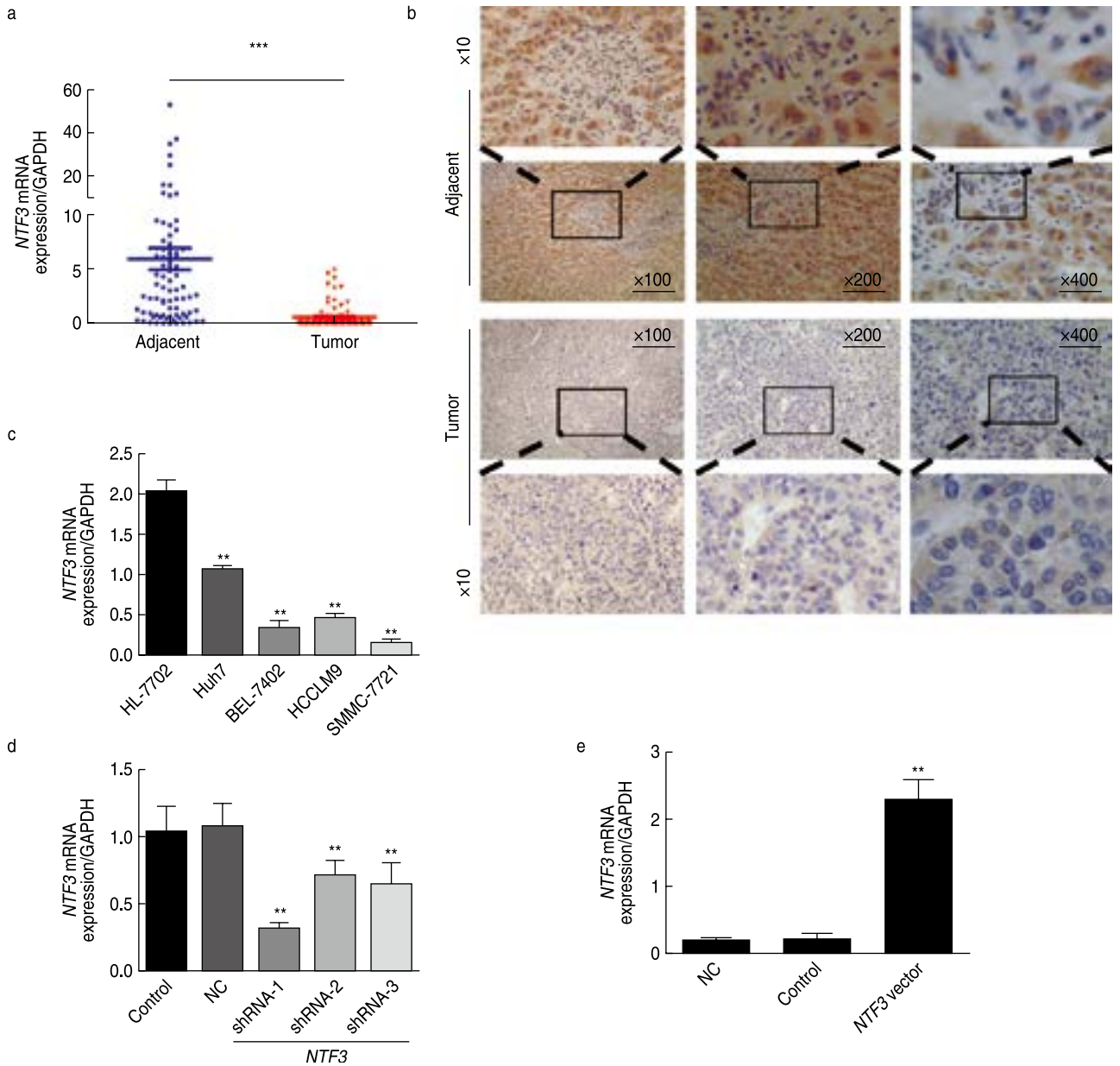

Fig. 2 *NTF3* is downregulated in HCC. (a) Box plot showing the expression of *NTF3* in HCC tumors and adjacent tissues; (b) Immunohistochemistry showing the expression of *NTF3* in tumors and adjacent tissues; (c) NTF3 expression in HCC cells; (d) *NTF3* mRNA expression in Huh7 cells following *NTF3* shRNA transfection; (e) *NTF3* mRNA expression in SMMC-7721 cells following transfection of an *NTF3* expression vector

different approaches. We found that upregulation of *NTF3* is strongly associated with decreased overall TNM stage and longer survival times. These results implicate *NTF3* in HCC pathogenesis and suggest its low expression is associated with the progression and metastasis of HCC. Moreover, our study proposed the targeting of *NTF3* as a potential treatment for HCC in addition to its possible use as a predictive marker of HCC outcomes in patients.

NTF3 has been suggested as a therapeutic target for breast cancer therapy [19]. Indeed, *NTF3* expression is increased in brain metastatic breast cancer cells and it has been demonstrated to promote the proliferation and metastasis of breast cancer cells in the brain by promoting the re-epithelialization of these cells and downregulating the microglial cytotoxic response [19]. In the present study, our results did not corroborate with these previous finding as we found that *NTF3* expression was decreased in HCC tissues and cells through different technical approaches (immunohistochemistry, Western blotting and qRT-PCR). These contradictory results may be due to the neuroprotective role of *NTF3* in the brain. In effect, the metastasis of cancer cells to the brain may

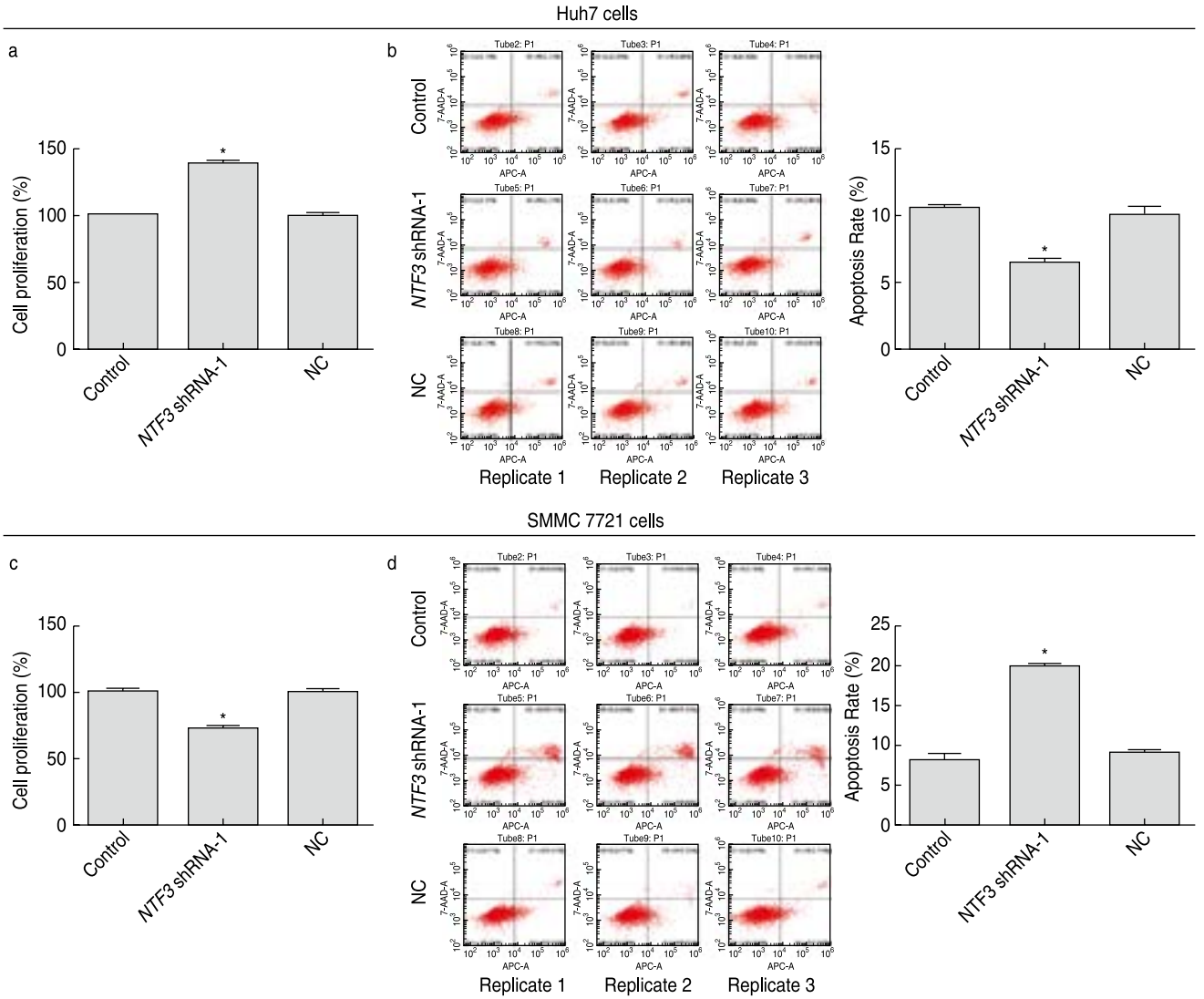

Fig. 3 *NTF3* inhibits proliferation and induce apoptosis of HCC cells. (a) Silencing of *NTF3* induces proliferation of Huh7 cells; (b) Silencing of *NTF3* hinders apoptosis in Huh7 cells; (c) Overexpression of NTF3 inhibits the proliferation of SMMC-7721 cells; (d) Overexpression of *NTF3* induces apoptosis in SMMC-7721 cells

induce the expression of *NTF3* in the brain, which could explain the increased expression of *NTF3* in the metastatic breast cancer cells in the brain. Our finding is the first to systematically demonstrate the downregulation of *NTF3* in HCC and its correlation with clinical characteristics. Our results imply that *NTF3* could play a significant role in HCC.

Previous studies have suggested that *NTF3* plays a functional role in the regulation of various cellular processes [19, 27–30]. However, the role of *NTF3* in HCC is still unclear. To uncover the function of *NTF3* in HCC, we silenced *NTF3* in Huh7 cells and found that it caused an increase in cell proliferation while inhibiting apoptosis. In addition, the overexpression of *NTF3* in SMMC-7721 cells was accompanied by decreased cell proliferation and increased apoptosis. Our results were contrary to those indicating that silencing of optineurin, which downregulates *NTF3* expression, increases apoptosis of RGC-5 cells [29] and that conditional knockdown of *NTF3* promotes neuronal apoptosis [30]. Similar results were reported for vascular smooth muscle cell proliferation [28]. Our results indicate that, despite its negative effect on apoptosis of various cells, *NTF3* induces the apoptotic cell death of HCC cells. Thus, we stipulated that *NTF3* could be used to kill cancer cells as a novel therapy.

Cell migration and invasion are critical processes involved in diverse physiological events as well as in the physiopathology of many disorders such as cancer [31–35]. Here, overexpression of *NTF3* inhibited the migration and invasion of HCC cells while contrary results were

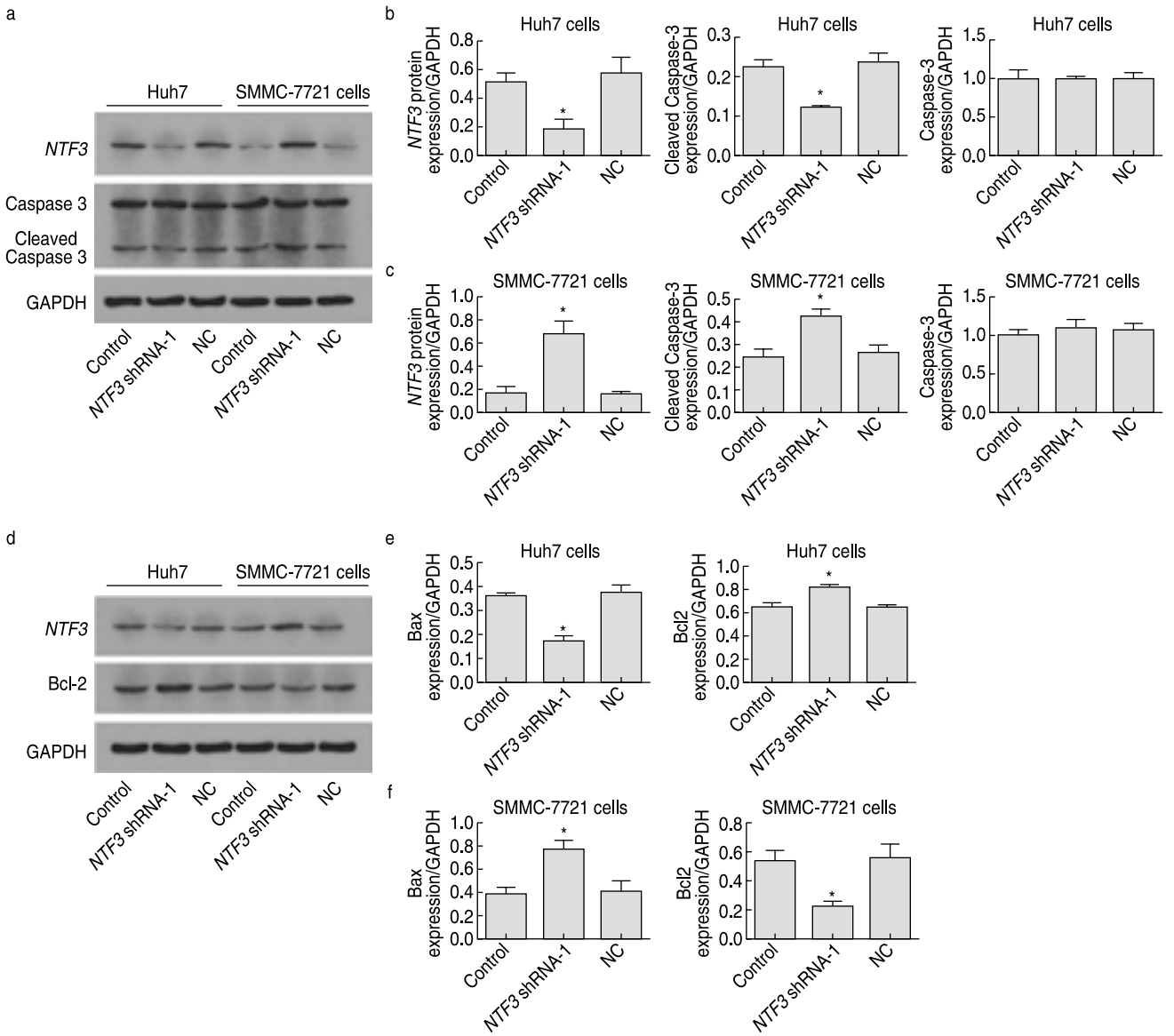

Fig. 4 *NTF3* regulates the expression of apoptosis markers in HCC cells. Western blot analysis of apoptosis markers was performed in Huh7 cells with silenced expression of *NTF3* and in SMMC-7721 cells

observed after *NTF3* silencing. Previous studies have indicated that SRY physically interacts with the *NTF3* promoter to synchronize cell migration in the testes during male sex determination [36]; however, the effect of *NTF3* on cell invasion has not been previously reported. Our study is the first to demonstrate that *NTF3* inhibits the migration and invasion of HCC cells. These results indicate that *NTF3* might inhibit the metastasis of HCC cells.

Conclusion

In this study, we examined the value of *NTF3* in HCC and demonstrated that (1) *NTF3* expression is decreased in HCC tissues and cells; (2) Decreased expression of *NTF3* is associated with a shorter survival time in HCC patients; (3) *NTF3* hinders proliferation, migration, invasion, and induces apoptosis of HCC cells. Owing to these results, we anticipate that *NTF3* might be a novel therapeutic target for HCC. However, further investigations are required for validating the effects of *NTF3* on the clinical course of HCC and on patient response to radiotherapy or chemotherapy.

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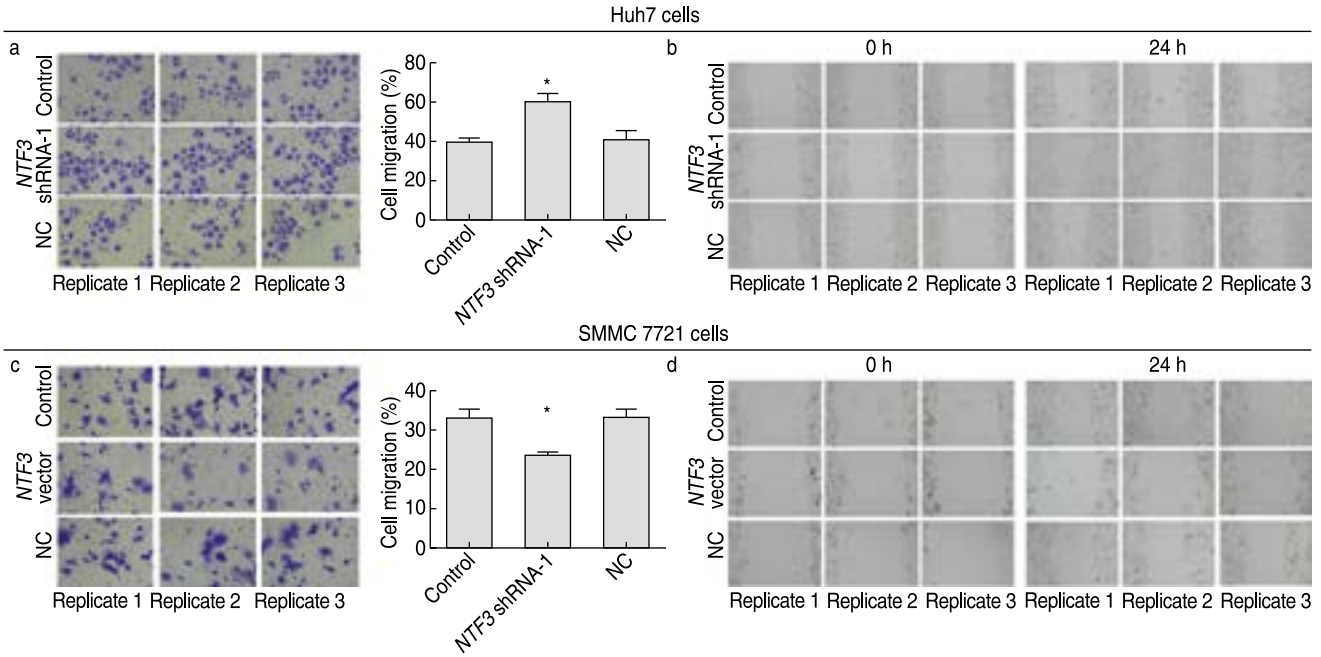

Fig. 5 *NTF3* inhibits invasion and migration of HCC cells. (a) Silencing of *NTF3* induces the invasion of Huh7 cells; (b) Silencing of *NTF3* induces the migration of Huh7 cells; (c) Overexpression of *NTF3* inhibits the invasion of SMMC-7721 cells; (d) Overexpression of *NTF3* inhibits the migration of SMMC-7721 cells

of Qingdao University, for advice on experimental design.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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