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Original Article

TrkC expression predicts favorable clinical outcome in invasive ductal carcinoma of breast independent of NT-3 expression

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Abstract: Background: TrkC, a member of neurotrophin receptor family, functions not only as an oncogene, but also act as a tumor suppressor via a manner of dependence receptor in human malignant tumors. Little is known on the action of TrkC for the clinical prognosis and the progression of breast cancer according to the availability of its ligand NT-3. We sought to investigate the prognostic relevance of NT-3-TrkC axis in breast cancer and estimate its role during the process of breast cancer progression. Methods: 236 cases of invasive ductal carcinoma (IDC), 60 pure ductal carcinoma in situ (DCIS) and 30 normal breast tissue (NBT) between 2004 and 2005 were included in the study. Spearman's rank correlation test was used to analyze the association of NT-3-TrkC expression and breast cancer progression. The Kaplan-Meier method and Cox proportional hazards model were performed to identify the relevant prognostic factors. Results: 50.4% IDC tumors displayed absent or low TrkC expression, while 49.6% was high TrkC expression. TrkC expression was negatively associated with lymph node metastasis (P = 0.029) and tumor proliferation (P = 0.015). Patients with lower TrkC expressing tumors had a higher risk of recurrence (odds ratio, 0.401; 95% confidence interval, 0.207-0.778; P = 0.007). The layered analysis indicated that patients with high TrkC expression tumors had a favor disease-free survival whether NT-3 and TrkC were co-expressed or solitarily expressed in the tumor (P = 0.000). NT-3 was demonstrated to be not a predictor of IDC patients' prognosis. But NT-3 expression was inversely correlated with the progression of breast cancer (r = -0.341, P = 0.000), since more IDC tumors showed high NT-3 expression than DCIS tumors (51.7% vs. 25.9%), while no NBT showed high NT-3 expression, as well. Conclusion: The study indicates TrkC expression reduces tumor relapse independent of NT-3 availability in the IDC. Elevated NT-3 expression contributes to the progression of breast cancer.

Keywords: TrkC, NT-3, invasive ductal carcinoma (IDC), breast, prognosis, progression, dependence receptor (DR)

Introduction

Tropomyosin-related kinase (Trk) family of neurotrophin receptors, TrkA, TrkB and TrkC, and their neurotrophin ligands has been previously studied extensively for their role in nervous system development, which contains intracellular tyrosine kinase activating domain. It is surprising that the Trk gene was then found as an oncogene or a proto-oncogene in many human cancers, including those with neuronal or non-neuronal origin. TrkA was an oncogenic fusion with tropomyosin gene and conferred constitutive activation of its tyrosine kinase activity to induce continuous proliferation of tumor cell [1-3]. Recently, a fusion of ETV6 to TrkC has also been reported to take place in acute leukemia and breast cancer [2, 4, 5]. In addition, Trk is also considered as the proto-oncogene which was shown to play a pleiotropic role in regulating important biology of many cancers. For example, the activation of tyrosine kinases induced by Trks and neurotrophins (NTs) binding has been reported to stimulate the proliferation and survival of a variety of tumor cells [6-10]. Trks may have a role in the progression of human cancers, depending on their types, despite the high homology among the three

kinds of Trks. It was reported that TrkC was the favorable marker in neuroblastoma (NB) and medulloblastoma (MBL) [11], while switching from TrkB to TrkC expression was necessary for the progression of medullary thyroid carcinoma [9]. A recent publication indicated TrkB was responsible for epithelial-mesenchymal transition in lung cancer and help to the progression of lung cancer [12].

At present, extensive work has been done for the role of nerve growth factor (NGF) and its high-affinity receptor TrkA in breast cancer. It has previously been shown that NGF stimulates the proliferation and survival of breast tumor cells through the activation of TrkA by a way of autocrine loop [13, 14]. And the anti-estrogen drug tamoxifen is able to inhibit the mitogenic effect of NGF [15]. However, there is a shortage of information on the action of TrkC and its preferential ligand NT-3, especially for the role of clinical patient's prognosis and the correlation between tumor progression and NT-3-TrkC axis. TrkC is reported to be overexpressed in breast cancers compared with normal tissues and in brain metastases of breast carcinomas [16, 17]. However, some reports found inconsistent results. Blasco-Gutiérrez suggested that elevated TrkC expression in breast invasive ductal carcinoma (IDC) indicated good prognosis since it was associated with lower grade tumors [16]. However, Korea authors presented in vitro evidences that TrkC overexpression could contribute to tumorigenesis, invasion and metastatic capability of the breast cancer cell [18]. Recent evidences have shown the possibility that TrkC, rather than functioning solely as oncogenes, may also, in at least some cases, act as tumor suppressor via a manner of dependence receptor (DR) in human malignant cancers [19, 20]. DR shares the ability to trigger apoptosis in the absence of their ligand, a feature that has been suggested to confer a tumor suppressor function to these receptors. There was evidence that the autocrine production of NT-3 by tumor cells constituted a selective growth advantage for some NB cells expressing TrkC [21].

Within the breast cancer research, NT-3 is almost undetectable and has never been a center of interest. Until now, no reports have evaluated the relation between NT-3-TrkC axis and prognosis, especially whether in a DR regulating manner. And will NT-3-TrkC expression contribute to the progression of breast cancer, from normal breast to in situ carcinoma, then to invasive carcinoma? In the present study, we investigate NT-3-TrkC expression in 236 cases of breast IDC samples; evaluate the association between NT-3-TrkC expression and the clinicopathological variables or patients' prognosis. We also estimate the role of NT-3-TrkC during the process of breast cancer progression.

Material and methods

Tissue specimens

Tissue collection and analysis in this study were approved by the Ethical Committee of Tianjin Medical University Cancer Institute and Hospital, China. Informed consent had been obtained from all patients before their surgery and the examination of the specimens were conducted. All cases of breast surgical specimens, fresh or paraffin-embedded, were anonymized after collection from the archival file of the Department of Breast Pathology, Tianjin Medical University Cancer Institute and Hospital.

A panel of fresh breast surgical specimens from 23 patients with invasive ductal carcinoma – not otherwise specified (IDC-NOS) was randomly selected in June 2013 for the further quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) and semi-quantitative RT-PCR test. For each sample, the tumor tissue was paired with the normal breast tissue 2 cm away from macroscopic cancer nodule. After the standard procedure for clinical diagnosis was completed, additional tissues were cut into small pieces, snap frozen in liquid nitrogen, and stored in a -80°C freezer until further use. Paraffin-embedded tissues of 236 IDC-NOS, 60 pure ductal carcinoma in situ (DCIS) and 30 normal breast tissues (NBT) 2 cm away from tumors were randomly selected from January 1st 2004 to December 30th 2005. All patients were women, ranging in age from 26 to 87 years (median 52 years). All IDC patients accepted surgery, including radial mastectomy (44/236, 18.6%), modified mastectomy (185/236, 78.4%) and breast-conserving surgery (7/236, 3.0%). The exclusion criteria were: bilateral breast cancer found at diagnose, patients treated with neoadjuvant chemotherapy and the patients lost to follow-up. The pathologic diagnosis had been confirmed by two senior

pathologists according to the 2003 WHO histological classification of tumors of the breast [22].

RNA extraction, semi-quantitative RT-PCR and quantitative RT-PCR

Fresh breast samples were snap frozen and pulverized in nitrogen liquid. Total RNA was isolated with TRIzol solution (Invitrogen, USA) according to the manufacturer's instructions. The RNA quality was controlled by formaldehyde gel electrophoresis. 1 μg of RNA was reverse-transcribed using the PrimeScript RT reagent Kit (TaKaRa, China). Regular PCR was done using a PrimeSTAR HS DNA Polymerase PCR kit according to the manufacturer's instructions (TaKaRa, China). PCR products were separated on 2.5% agarose gels and visualized by ethidium bromide. Q-RT-PCR was performed on a CFX96 apparatus (Bio-Rad) using the SYBR Premix Ex Taq™ IIkit (TaKaRa, China). Polymerase was activated at 95°C for 30 seconds, followed by the subsequent two steps PCR conditions, which were 40 cycles at 95°C for 30 seconds and 60°C for 20 seconds. Data were analyzed using the MX4000 PCR system software (USA). The sequences of the following primers are: human NT-3, forward 5'-CATGTCGACGTCCCTGGAA-3' and reverse 5'-CCTTGGATGCCACGGAGATAA-3'; human TrkC, forward 5'-CTATCACTGTGACCCACAAACCAGA-3' and reverse 5'-CAAATTTGGACCGTCGACCATA-3'; human GAPDH, forward 5'-TGCACCACCAACTGCTTAGC-3' and reverse 5'-GGCATGGACTGTGGTCATGAG -3', which was used as a reference gene.

Immunohistochemical procedures and evaluation

Immunohistochemistry (IHC) was performed by using the labeled streptavidin-biotin technique, with antibodies against TrkC, NT-3, Ki67, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) in all cases, and cytokeratin (CK)5/6 and epidermal growth factor receptor (EGFR) only in ER-/PR-/HER2- cases. Briefly, 4 μm sections of tumor tissue were deparaffinized in xylene and hydrated in a graded series of alcohols. Antigen retrieval was performed using a pressure cooker in citrate buffer (pH 6.0) or EDTA (pH 9.0) solution. Following incubation in 3% H_2O_2 for 10 min, inactivating the endogenous peroxidase activity, the sections were treated with a blocking solution containing 10% normal goat serum for 20 min at room temperature. Then tissue sections were incubated with primary antibodies at 4°C overnight. Primary antibodies used in this study included TrkC (1:100; Santa Cruz, USA), NT-3 (1:300; Santa Cruz, USA), Ki-67 (1:75; Zymed, USA), ER (1:150; Zymed, USA), PR (1:150; Zymed, USA), anti-HER-2/neu (1:100; Invitrogen), CK5/6 (1:200; Invitrogen) and EGFR (1:100; Zymed, USA). After incubation with anti-mouse or rabbit biotin-conjugated secondary antibody and streptavidin-horseradish peroxidase (Zymed) for 30 min at 37°C, respectively, color was developed by incubation with 3, 3'-diaminobenzidine tetra-hydrochloride (DAB). The sections were counterstained with hematoxylin. Sections with normal lobules of mammary gland adjacent to tumor were used as an auto-specific positive control for ER and PR. And sections of the invasive ductal carcinomas that were positive for HER2, Ki-67, CK5/6 and EGFR were used each time as a positive control. As a negative control, the primary antibody was replaced with normal mouse or rabbit immunoglobulin.

The immunostaining was scored in double blind by two senior pathologists, who were blinded to patients' clinicopathologic characteristics and outcomes. TrkC was expressed in the cytoplasm and/or membrane and NT-3 was expressed in the cytoplasm. Protein expression was semi-quantified according to the immunoreactivity score (IRS) developed by Remmele and Schicketanz [24], which is the product of immunoreactivity intensity (0, no reactivity; 1, weak reactivity; 2, moderate reactivity; 3, strong reactivity) and percentage of positive cells (0, 0% of cells reactive; 1, < 25% of cells reactive; 2, 25-75% of cells reactive; 3, ≥ 75% of cells reactive). Each case was graded by the addition of the two scores as 0 (—), 1-2 (+) and 3-4 (++) and 5-6 (+++). For ER, PR, the location of immunoreactivity, percentage of stained cells, and intensity were determined. Ki67 positive stains were nuclear staining and Ki67 status was expressed in terms of percentage of positive cells, with a threshold of 14% of positive cells. ER+ or PR+ was defined as > 1% of the tumor cells presented nuclear staining with different degrees. A positive HER2 result was IHC staining of 3+ (uniform, intense membrane staining of > 30% of invasive tumor cells); a negative result is an IHC staining of 0 or 1+;

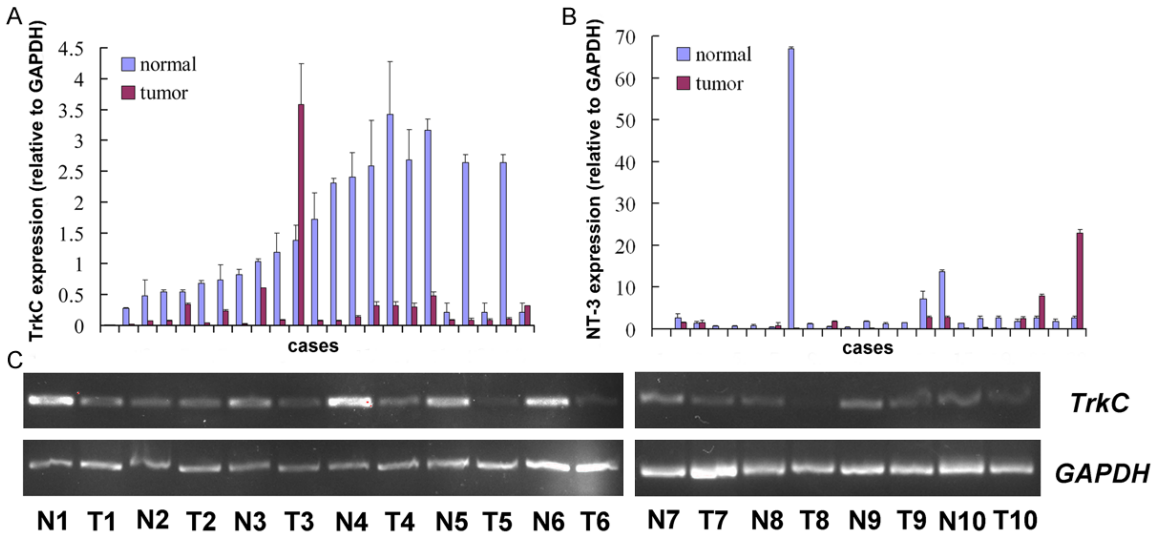

Figure 1. TrkC and NT-3 mRNA expression in invasive ductal carcinoma (IDC) and corresponding normal fresh tissues. (A, B) Quantitative real-time RT-PCR was performed using total RNA extracted from normal and tumoral tissues with specific human TrkC and NT-3 primers. The expression levels in 23 beast IDC tumors and corresponding normal tissue are given as a ratio between TrkC (A) or NT-3 (B) and GAPDH, the internal control. (C) The electrophoresis graph of regular RT-PCR displayed TrkC mRNA expression from 10 representative pairs of IDC and corresponding normal breast tissues. GAPDH was used as a housekeeping gene.

while an equivocal result was an IHC staining of 2+. CK5/6 and EGFR stains were considered positive if any cytoplasmic and/or membranous staining was observed.

Follow-up study

Follow-up data were obtained via medical archives, mails, telephone calls and study questionnaire. Survival periods were calculated from the time of surgery, and a median follow-up period was 59 months (range, 19-99 months). The follow-up contacts were carried out at 3-month intervals over the first year, 6-month intervals during the second year and at 12-month intervals thereafter. Relapse was defined as radiographic or pathological evidence of regional tumor recurrence or distant metastasis at any time after initial therapy. Primary end points of the study were overall survival (OS) and disease-free survival (DFS).

Statistical analysis

The *t* test of two independent-samples was used to compare the TrkC and NT-3 transcription level between the fresh paired normal breast and breast cancer tissues. As for paraffin-embedded tissues, the differences of TrkC and NT-3 expression in NBT, DCIS and IDC samples, or the association of NT-3-TrkC expression with clinicopathological features were evaluated using Chi-square test. And correlation of NT-3-TrkC expression among NBT, DCIS and IDC were evaluated with Spearman's rank correlation test. For univariable survival analysis, overall survival (OS) and disease-free survival (DFS) rates were estimated by the Kaplan-Meier curves. The log-rank test was used to compare survival differences between the tumors with different NT-3-TrkC expression. Cox proportional hazards regression model was performed for the identification of relevant prognostic factors. All statistical tests were two-sided at the 5% level of significance and were performed using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA).

Results

Expression of TrkC and NT-3 transcription in human breast cancers

According to Q-RT-PCR's results, TrkC expression displayed a markedly decreased expression in tumors (P = 0.000, **Figure 1A**, **1B**). However, NT-3 expression didn't show significant differences between the tumors and their matched normal tissues (P = 0.164). As indicated in Supplementary Table 1, an 8-fold decrease of TrkC expression was observed in over 50% of the tested tumors, and about one-

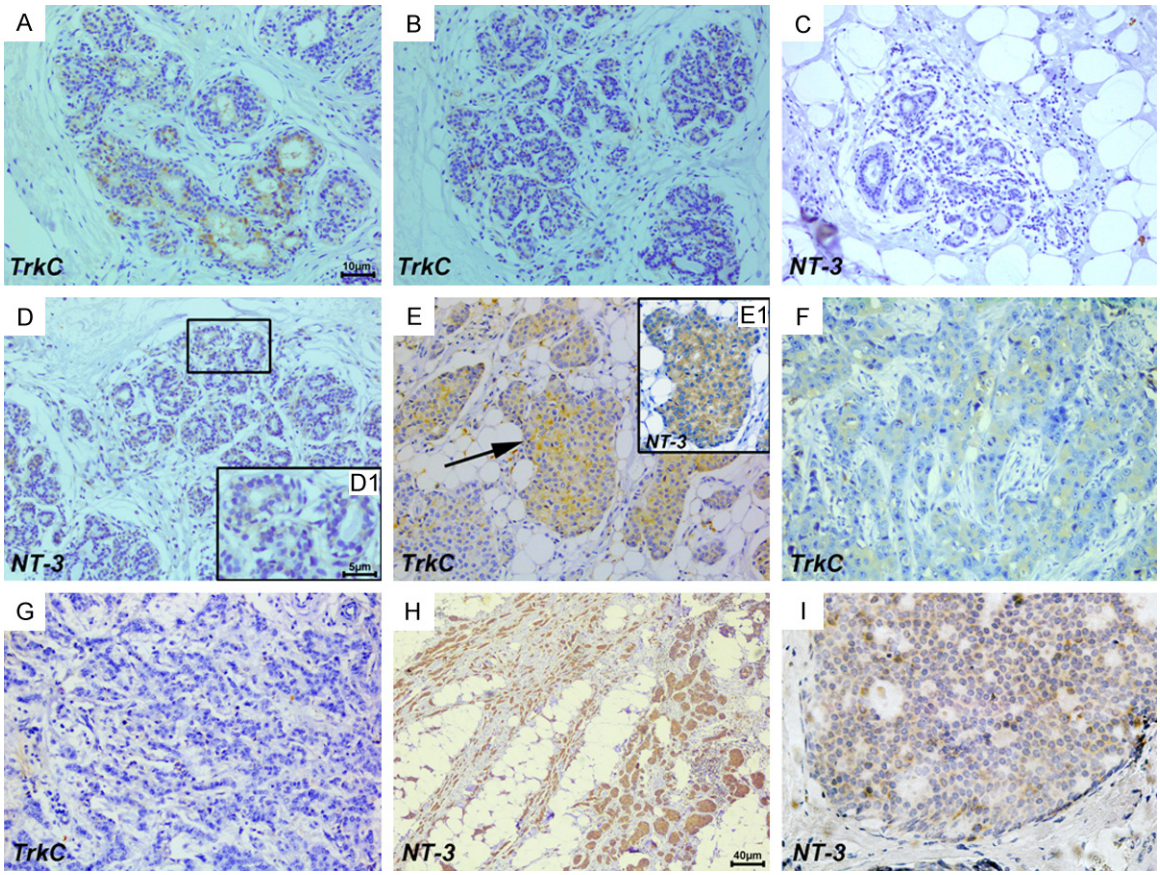

Figure 2. Representative immunostaining of TrkC and NT-3 in normal breast tissue (NBT), invasive ductal carcinoma (IDC) and ductal carcinoma in situ (DCIS) samples. Moderate staining (A) or weak staining (B) of TrkC was found in the normal breast lobule. But NT-3 expression was usually absent (C) or weak (D). D1 was the magnified image of the boxed area in (D). TrkC and NT-3 expression in IDC is not always uniform. Tumor cells in IDC displayed strong staining (E), weak staining (F) or no staining (G) of TrkC. Sometimes TrkC (E) and NT-3 (E1) proteins are strongly co-expressed in the invasive cancer cells (arrow indicated the same cancer nest in e as the boxed image of E1). (H) Invasive NT-3 strongly positive cancer cells infiltrate into the stroma and adipose tissue of the breast IDC. (I) Moderate staining of NT-3 was detected in DCIS. Scale bars = 5 μm in (D1, I), 10 μm in (A-G, E1), and 40 μm in (H).

fifth of the tumors showed an approximate 20-fold decrease in expression. Mean TrkC expression was more than 4-fold lower in neoplasms than in the corresponding normal tissues. Result of regular RT-PCR verified Q-RT-PCR's result (**Figure 1C**). TrkC and NT-3 expression in breast IDC were found to be independent of the clinicopathological characteristics of patients, such as age, pathological tumor size, tissue grade, lymph node metastasis and receptor status (data not shown).

Expression of TrkC and NT-3 in human breast IDC-NOS, DCIS and NBT

TrkC was obviously expressed in the cytoplasm and cytomembrane of luminal epithelium or cancer cell, while NT-3 was mainly expressed in the cytoplasm (**Figure 2**). Most of NBT cases showed weak or medium expression of TrkC (73.3%), and no strong TrkC expression was detected. For NT-3 expression, 80% NBT cases displayed negative expression, while 20% was weak positive (**Figure 2A-D**, **Table 1**). But in the IDC tumors, TrkC and NT-3 were sometimes strongly co-expressed in the same invasive cancer cells (**Figure 2E**, **2E1**), while weak or absent TrkC expression was often detected (**Figure 2F**, **2G**). In contrast to NBT, TrkC and NT-3 expression in IDC-NOS and DCIS was not always consistent (**Table 1**). As **Table 2** indicated, more IDC tumors showed high NT-3 expression than DCIS cases (51.7% vs. 25.9%), and no NBT cases showed high NT-3 expression, as well. Moreover, NT-3 expression was negatively associated with the progression of breast can-

Table 1. TrkC and NT-3 expression IDC, DCIS and NBT

TrkC expression, No (%)		NT-3 expression, No (%)				
		—	+	++	+++	Total
IDC (n = 236)	—	20 (31.2)	14 (21.9)	18 (28.1)	12 (18.8)	64 (100)
	+	14 (25.5)	14 (25.5)	16 (29.1)	11 (20.0)	55 (100)
	++	7 (8.8)	26 (32.5)	18 (22.5)	29 (36.2)	80 (100)
	+++	8 (21.6)	11 (29.7)	7 (18.9)	11 (29.7)	37 (100)
	Total	49 (20.8)	65 (27.5)	59 (25.0)	63 (26.7)	236 (100)
DCIS (n = 60)	—	6 (35.3)	3 (17.6)	4 (23.5)	4 (23.5)	17 (100)
	+	2 (28.6)	5 (71.4)	0 (0)	0 (0)	7 (100)
	++	8 (53.3)	0 (0)	5 (33.3)	2 (13.3)	15 (100)
	+++	5 (23.8)	2 (9.5)	10 (47.6)	4 (19.0)	21 (100)
	Total	21 (35.0)	10 (16.7)	19 (31.7)	10 (16.7)	60 (100)
NBT (n = 30)	—	5 (62.5)	3 (37.5)	/	/	8 (100)
	+	13 (92.9)	1 (7.1)	/	/	14(100)
	++	6 (75.0)	2 (25.0)	/	/	8 (100)
	Total	24 (80.0)	6 (20.0)	/	/	30 (100)

Table 2. The association of TrkC and NT-3 expression among IDC, DCIS and NBT samples

Protein expression	IDC (n=236 cases), No (%)	DCIS (n=60 cases), No (%)	NBT (n=30 cases), No (%)	χ^2	P^a	r	P^b
TrkC				8.903	0.012	-0.069	0.214
— or +	119 (50.4)	24 (40.0)	22 (73.3)				
++ or +++	117 (49.6)	36 (60.0)	8 (26.7)				
NT-3				37.942	0.000	-0.341	0.000
— or +	114 (48.3)	45 (75.0)	30 (100)				
++ or +++	122 (51.7)	15 (25.0)	0 (0)				

[a]Chi-square test and [b]Spearman's rank correlation test are used. *P*-value ≤ 0.05 are considered statistically significant.

cer, from NBT to DCIS to IDC (r = -0.341, P < 0.05, **Table 2**). When multiple comparisons among NBT, DCIS and IDC cases were performed, we found significant differences still exited (P < 0.017). However, TrkC expression seemed to have no correlation with this progression (P > 0.05).

Association of NT-3-TrkC expression with clinicopathological variables and prognosis in IDC patients

TrkC and NT-3 expression in 236 cases of IDC tumors were classified into two major subgroups: negative or weak expression and medium or strong expression, which were referred as "—/+" and "++/+++", respectively. As indicated by **Table 3**, with elevated TrkC expression, more IDC cases had no lymph node metastasis verified by pathological evidences (59.8% vs. 44.5%). A negative association between TrkC expression and lymph node metastasis was indicated (χ^2 = 5.528, P = 0.029). Meanwhile, TrkC expression was inversely associated with Ki67 proliferation index (χ^2 = 5.975, P = 0.015). No correlation was found between TrkC expression and age, tumor size and grade, stage, receptor status and molecular subtypes, and postoperative therapy means. However, there was no correlation of NT-3 expression with all the indicated clinicopathological variables in Supplementary Table 2.

It seemed that more recurrence events happened in the patients with decreased TrkC expression (36 cases (30.3%) vs. 17 cases (14.5%)), as compared with high TrkC expression. And the number of death cases in the patients with low and high TrkC expression was 13 cases (10.9%) and 11 cases (9.4%), respectively at the end of following-up period (**Table 4**). An obviously different DFS existed in the IDC tumors with different TrkC expression (χ^2 =

Table 3. The association between TrkC expression and clinicopathological characteristics in IDC

Characteristics	TrkC expression		χ^2	*P*
	—/+, No (%)	++/+++, No (%)		
Age			4.121	0.127
< 40	15 (12.6)	6 (5.1)		
40-50	40 (33.6)	41 (35.0)		
> 50	64 (53.8)	70 (59.8)		
Pathological tumor size			2.463	0.292
≤ 2 cm	38 (31.9)	42 (35.9)		
> 2 cm, ≤ 5 cm	73 (61.3)	72 (61.5)		
> 5 cm	8 (6.7)	3 (2.6)		
Tumor grade			1.692	0.429
1	20 (16.8)	23 (19.7)		
2	66 (55.5)	55 (47.0)		
3	33 (27.7)	39 (33.3)		
Lymph node status				
Negative	53 (44.5)	70 (59.8)	5.528	0.029
Positive	66 (55.5)	47 (40.2)		
AJCC stage			3.717	0.156
I	18 (15.1)	29 (24.8)		
II	70 (58.8)	64 (54.7)		
III	31 (26.1)	24 (20.5)		
ER			0.000	0.994
Negative	56 (47.1)	55 (47.0)		
Positive	63 (52.9)	62 (53.0)		
PR			0.060	0.806
Negative	51 (42.9)	52 (44.4)		
Positive	68 (57.1)	65 (55.6)		
HER2			3.205	0.201
Negative	95 (79.8)	93 (79.5)		
Positive	12 (10.1)	18 (15.4)		
Equivocal	12 (10.1)	6 (5.1)		
Ki-67			5.975	0.015
≤ 14%	23 (19.3)	39 (33.3)		
> 14%	96 (80.7)	78 (66.7)		
Molecular subtypes			0.351	0.950
Luminal A	56 (47.1)	59 (50.4)		
Luminal B	17 (14.3)	17 (14.5)		
HER2	11 (9.2)	10 (8.5)		
Triple negative	35 (29.4)	31 (26.5)		
Chemotherapy			0.002	0.965
Yes	107 (89.9)	105 (89.7)		
No	12 (10.1)	12 (10.3)		
Radiotherapy			0.273	0.602
Yes	32 (27.1)	28 (24.1)		
No	86 (72.9)	88 (75.9)		
Endocrine therapy			2.058	0.151
Yes	55 (46.2)	65 (55.6)		
No	64 (53.8)	52 (44.4)		

Chi-square test is used. *P*-value ≤ 0.05 are considered statistically significant.

10.272, $P = 0.001$). A more prolonged DFS were found in IDC tumors with high TrkC expression ($P \leq 0.05$). However, OS was not significant different with different TrkC expression ($\chi^2 = 0.408$, $P = 0.523$) (**Figure 3A**, **3B**, **Table 5**). However, there was no difference of DFS and OS in the tumors with different NT-3 expression (**Figure 3C**, **3D**). Considering TrkC receptor may play a totally inverse role for the behavior of tumor cell based on its ligand-NT-3 expression or not, we analyzed the survival in a layered comparison, according to whether NT-3 and TrkC were co-expressed or solitarily expressed in the tumor. Nevertheless, the layered analysis did not affect the results, whereby elevated TrkC expression still correlated with a favor DFS but not OS, whether NT-3 and TrkC were co-expressed or solitarily expressed in IDC tumors (**Figure 4**). And no correlation between NT-3 expression and the survival was found as well in the layered analysis in Supplementary Figure 1. Multivariable Cox analysis revealed that age > 50 years, lymph node positive, negative or weak TrkC expression, without radiotherapy, without endocrine therapy, HER2 overexpression and triple negative breast cancer subtypes were risk predictors of relapse ($P \leq 0.05$), while tumor grade 3, triple negative breast cancer subtypes, and without chemotherapy were risk predictors of death ($P \leq 0.05$). TrkC expression in tumors was demonstrated to be an independent risk factor for relapse, but not for death (**Table 5**).

Discussion

TrkC is a neurotrophin receptor that belongs to the receptor tyrosine kinase (RTK) family and has been reported to play key roles in the processes of many human solid tumors including breast carcinoma as an oncogene or a proto-oncogene [21, 24]. TrkC has been involved in oncogenic translocations t(12;15) in secretory breast carcinoma, resulting a ETV6-NTRK3 chimeric protein, which causes malignant transfor-

Table 4. The association of relapse and survival events, mean DFS and OS time and 95%CI with NT-3-TrkC expression in 236 cases of IDC

	DFS				OS			
	No relapse No (%)	Relapse No (%)	Mean (months)	95% CI (months)	Survival No (%)	Death No (%)	Mean (months)	95% CI (months)
TrkC expression								
—/+	83 (69.7)	36 (30.3)	67.064	61.794-72.334	106 (89.1)	13 (10.9)	89.939	85.255-94.624
++/+++	100 (85.5)	17 (14.5)	86.243	81.923-90.564	106 (90.6)	11 (9.4)	89.940	86.563-93.317
NT-3 expression								
—/+	90 (78.9)	24 (21.1)	79.784	73.938-85.630	104 (91.2)	10 (8.8)	92.616	88.821-96.412
++/+++	93 (76.2)	29 (23.8)	78.889	73.421-84.357	108 (88.5)	14 (11.5)	87.382	83.175-91.588

CI: confidence interval, DFS: disease-free survival, IDC: invasive ductal carcinoma, OS: overall survival.

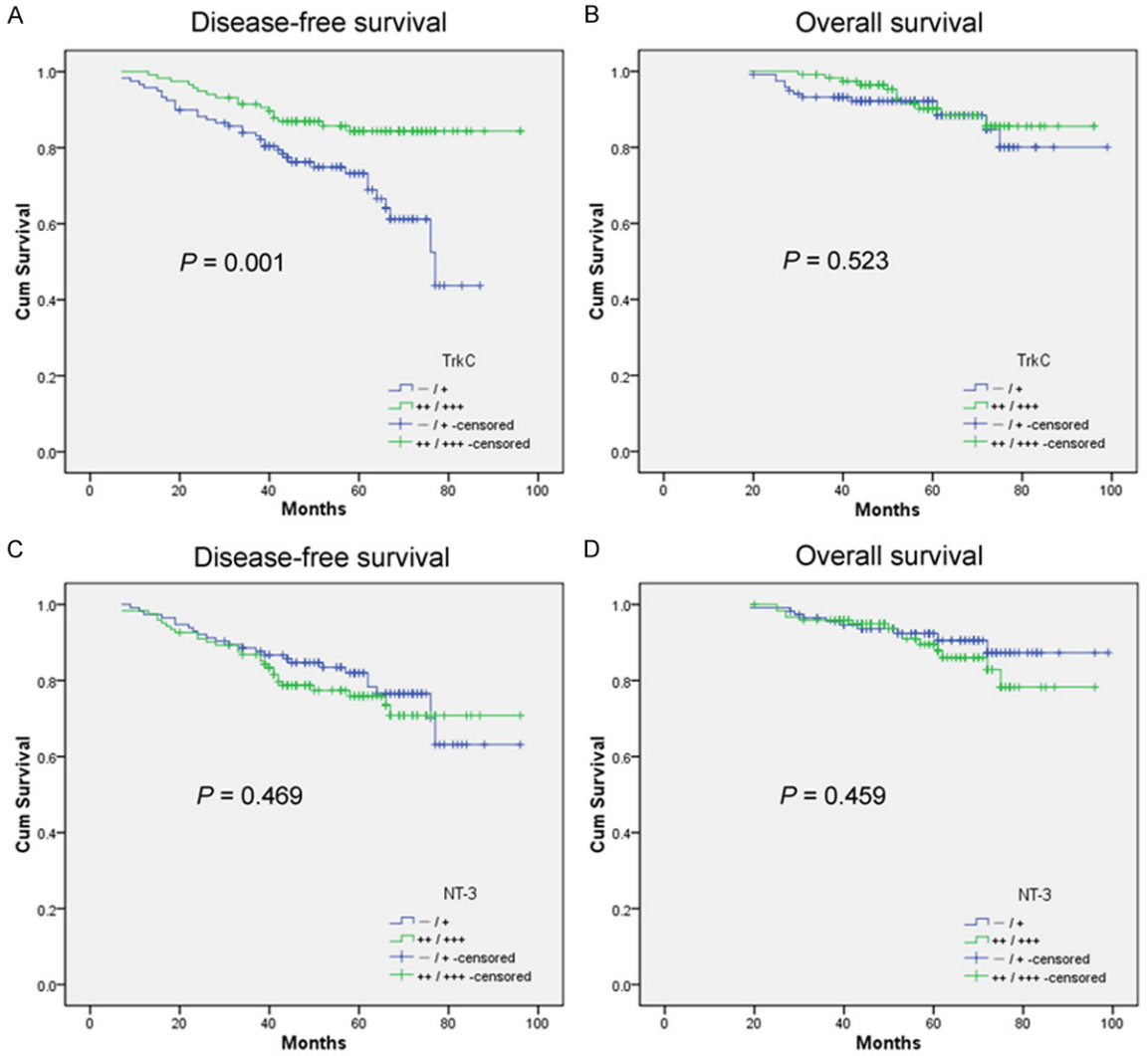

Figure 3. Kaplan-Meier curves of disease free survival (DFS) and overall survival (OS) for invasive ductal carcinoma (IDC) patients with different TrkC or NT-3 expression. A, B. DFS and OS curves of IDC patients according to the TrkC immunostaining. C, D. DFS and OS curves of IDC patients according to the NT-3 immunostaining.

mation of normal epithelial cell [4, 5]. However, ETV6-NTRK3 gene fusion was only confirmed in the secretory breast carcinoma, but not in other ductal carcinomas. Many scholars have ob-

Table 5. Multivariate analysis of risk factors for relapse and death

Characteristics	Relapse (n = 53 cases)			Death (n = 24 cases)		
	HR	95% CI	*P*-values	HR	95% CI	*P*-values
Age						
< 40	1			1		
40-50	3.210	1.834-12.350	0.190	0.618	0.122-3.639	0.640
> 50	6.460	1.720-24.265	0.026	3.115	0.657-14.768	0.152
Pathological tumor size						
≤ 2 m	1			1		
> 2 cm, ≤ 5 cm	1.503	0.568-3.980	0.412	0.449	0.142-1.420	0.173
> 5 cm	2.084	0.494-8.800	0.318	0.548	0.080-3.774	0.542
Tumor grade						
1	1			1		
2	1.049	0.447-2.462	0.912	1.313	0.329-5.247	0.700
3	1.617	0.598-4.369	0.344	3.592	3.895-5.416	0.032
Lymph node status						
Negative	1			1		
Positive	1.975	1.125-3.466	0.018	2.085	1.575-3.561	0.024
AJCC stage						
I	1			1		
II	0.791	0.223-2.808	0.717	0.944	0.157-5.672	0.950
III	2.286	0.345-15.143	0.319	2.574	0.267-24.789	0.413
TrkC						
—/+	1			1		
++/+++	0.401	0.207-0.778	0.007	0.552	0.231-1.321	0.182
NT-3						
—/+	1			1		
++/+++	1.407	0.780-2.536	0.257	1.588	0.695-3.629	0.272
Ki-67						
≤ 14%	1			1		
> 14%	0.910	0.430-1.922	0.804	1.208	0.447-3.261	0.710
Molecular subtypes						
Luminal A	1			1		
Luminal B	1.969	1.388-3.996	0.414	1.226	0.732-2.126	0.212
HER2	2.256	1.066-4.666	0.046	2.033	1.125-4.758	0.123
Triple negative	1.948	1.274-3.863	0.010	2.274	1.452-5.532	0.028
Chemotherapy						
Yes	1			1		
No	1.617	0.521-5.023	0.406	3.634	2.708-10.195	0.044
Radiotherapy						
Yes	1			1		
No	2.291	1.917-4.725	0.036	0.533	0.176-1.610	0.264
Endocrine therapy						
Yes	1			1		
No	3.292	2.113-5.754	0.011	2.470	1.141-3.566	0.219

Cox proportional hazards regression analysis is used. *P*-value ≤ 0.05 are considered statistically significant. AJCC: American Joint Committee on Cancer, CI: confidence interval.

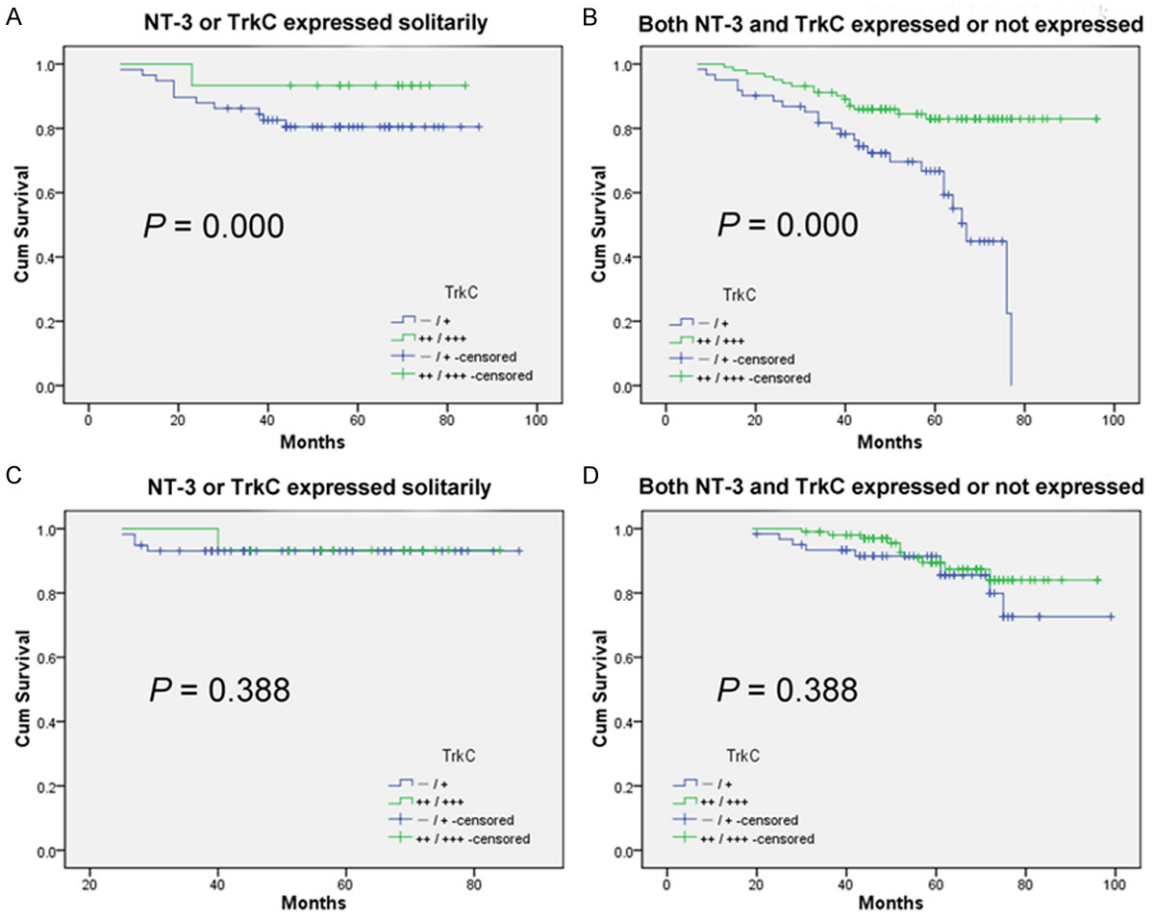

Figure 4. The survival analysis for invasive ductal carcinoma patients with different TrkC expression in a layered comparison based on the correlation of NT-3 and TrkC expression. Whether in the solitary expression of NT-3 or TrkC (A, C) or in the co-expression of NT-3 and TrkC (B, D), elevated TrkC expression was associated with an obvious prolonged disease free survival (DFS) (A, B) ($P < 0.01$), but didn't correlate with overall survival (OS) (C, D) ($P > 0.05$).

served the TrkC expression in breast carcinoma. However, its role in the process of carcinogenesis and development is not clear because it is difficult to achieve uniform results. TrkC is reported to be overexpressed in breast cancers compared with normal tissues and in brain metastases of breast carcinomas [16-18]. However, there was one report that indicated TrkC and its preferential ligand NT-3 were not detected in breast cancer cells and tumor biopsies [25]. In the current study, TrkC and NT-3 expressed in the IDC tumor, but their expression always varied. Almost half of tumors (50.4% vs. 48.3%) displayed absent or low expression of TrkC and NT-3, and another half (49.6% vs. 51.7%) showed high expression, respectively (**Table 1**). The cause of these contradicting results may be derived from the use of polyclonal antibody, since TrkC monoclonal antibody has not been used in the study of breast carcinoma till now, which may produces cross response with other Trk receptors. Our Q-RT-PCR results indicated that TrkC mRNA expression decreased in most IDC tumors when compared with their corresponding normal tissue. The TrkC mRNA expression in the most of tumors (approximately 91%) was lower than that in the normal breast (Supplementary Table 1), which may be different from the immunohistochemistry's results. Jin's reports found a similar phenomenon that 84% tumor showed elevated TrkC protein expression detected by western blot means in collected 38 cases of IDC, but not for the TrkC mRNA level, when compared with normal breast tissue [18]. We speculated that different post-transcriptional regulating TrkC mechanisms happened during the process of breast cancer tumorigenesis.

Except for acting as an oncogene, TrkC has several characteristics of a tumor suppressor in

some solid tumors [19, 21]. And TrkC expression was found as an indicator of good prognosis in invasive breast carcinoma, since its expression was inversely correlated with tumor grade [17]. We showed that TrkC transcription expression was down-regulated in most of human breast cancers, which suggest TrkC may act as a tumor suppressor. With elevated TrkC expression, more IDC patients had no lymph node metastasis and more IDC tumor displayed low Ki67 proliferation. This phenomenon suggests IDC tumor with high TrkC expression may have the capability of low invasion and proliferation. The survival analysis verified this result, whereby TrkC expression was positively correlated with longer DFS. And multivariable Cox analysis indicated TrkC expression in IDC tumors was independent risk factors for relapse, but not for death (**Table 5**). TrkC expression was often lost in human breast cancer cell lines, such as MDA-MB-231, MCF-7, T47D et al, which was confirmed by Jin's and our studies [18]. The absent TrkC expression may be another growth advantage for breast cancer cells. However, Jin et al. found TrkC overexpression could contribute to tumorigenesis, invasion and metastatic capability of the breast cancer cell, which may be contradictory to our results [18]. We speculate that TrkC expression in breast cancer of different species may result in this inconsistency, since they primary concentrate on in vitro studies and use mice-derived breast cancer cells. And the comparative study among the breast cancer cells from different species may solve this problem.

As a DR, TrkC can initiate two completely opposite signaling pathways depending on NT-3 availability: a positive differentiation and survival signal, or an active death signal. TrkC can induce caspase-mediated apoptotic death in the absence of NT-3 in a variety of immortalized cells, including those of non-neuronal origin [19, 20]. A selective advantage for a tumor cell to survive in an environment with limited ligand availability would hence be either to lose the expression of the dependence receptor, or to gain expression of its ligand. Bouzas-Rodriguez found the antitumoral role was inhibited in a faction of aggressive neuroblastoma by autocrine expression of NT-3. Meanwhile, disruption of the autocrine production of NT-3, either by siRNA or by inhibition of NT-3-TrkC interaction, induced apoptosis in NT-3 expressing neuroblastoma cells [21]. However, human colorectal cancer cells display another mechanism allowing them to bypass TrkC's novel antitumoral control: TrkC expression is often silenced by promoter methylation in a large faction of human colorectal cancers [19]. We found both negative and positive NT-3 expression was detected in IDC tumors (**Table 1**). Depending on whether NT-3 and TrkC were co-expressed or solitarily expressed in the tumor, the layered analysis did not affect patients' survival, whereby elevated TrkC expression still correlated with a favor survival in IDC patients (**Figure 4**). We guess several reasons may result in this phenomenon. Bardelli et al found TrkC displays a large spectrum of missense mutations in sporadic colorectal cancers, covering the entire TrkC coding sequence, including pro-apoptotic and kinase coding region, which may result in oncogenic-gain-of-function mutations or loss-of-proapoptotic-function mutations [20, 26]. Till now, there has no similar systemic mutation analysis of TrkC receptor in breast carcinoma. But we believed the frequent mutation of TrkC receptor in malignant tumors made it difficult to elucidate its role in breast carcinoma. In addition, analysis for the expression of different TrkC isoforms in breast carcinoma has the possibility to solve this problem, since the truncated isoforms may lose the intracellular tyrosine kinase activity.

In the IDC tumors, there was no correlation of NT-3 expression with all the indicated clinicopathological parameters and patients' prognosis in Supplementary Table 2. However, elevated NT-3 expression contributes to the progression of breast cancer from NBT to in situ carcinoma, then to invasive carcinoma, while TrkC expression was not associated with this progression. Classic activation of TrkC occurs by receptor multimerization in response to its ligand NT-3 binding, which further activate two primary downstream PI3K/Akt and Ras/MEK/MAPK signaling pathways. These two pathways are well-known to be important for the tumorigenesis and progression of breast cancer [27]. Although we don't know why NT-3 doesn't correlate with the invasion and metastasis of IDC tumor, we speculated classic RTK mediated pathways upon NT-3-TrkC binding might be a key factor during the process of breast cancer genesis and development. And a systemic study via cell model is needed to elucidate the phenomenon in the future.

In conclusion, our study indicates aberrant NT-3-TrkC autocrine loop is a key factor for the pathogenesis and progression of breast cancer. In the breast IDC, high TrkC expression may predict low invasiveness and proliferation of the tumor. And TrkC expression is demonstrated to be an independent prognostic factor for relapse. Although NT-3 expression does not correlate with IDC patients' prognosis, elevated NT-3 expression is associated with the progression of breast cancer from NBT to DCIS, then to IDC. The intervention must target on the different molecules of NT-3-TrkC axis at different phases of breast cancer pathogenesis and development. In addition, TrkC may act as a tumor suppressor in the IDC tumor independent of NT-3 availability, which does not exclude the possibility that it still play the role via a manner of DR. A systemic in vitro study and mutation analysis of TrkC receptor in breast cancer help to elucidate this problem.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. TrkC expression level in human breast tumor samples and its expression ratio compared with the corresponding normal tissue

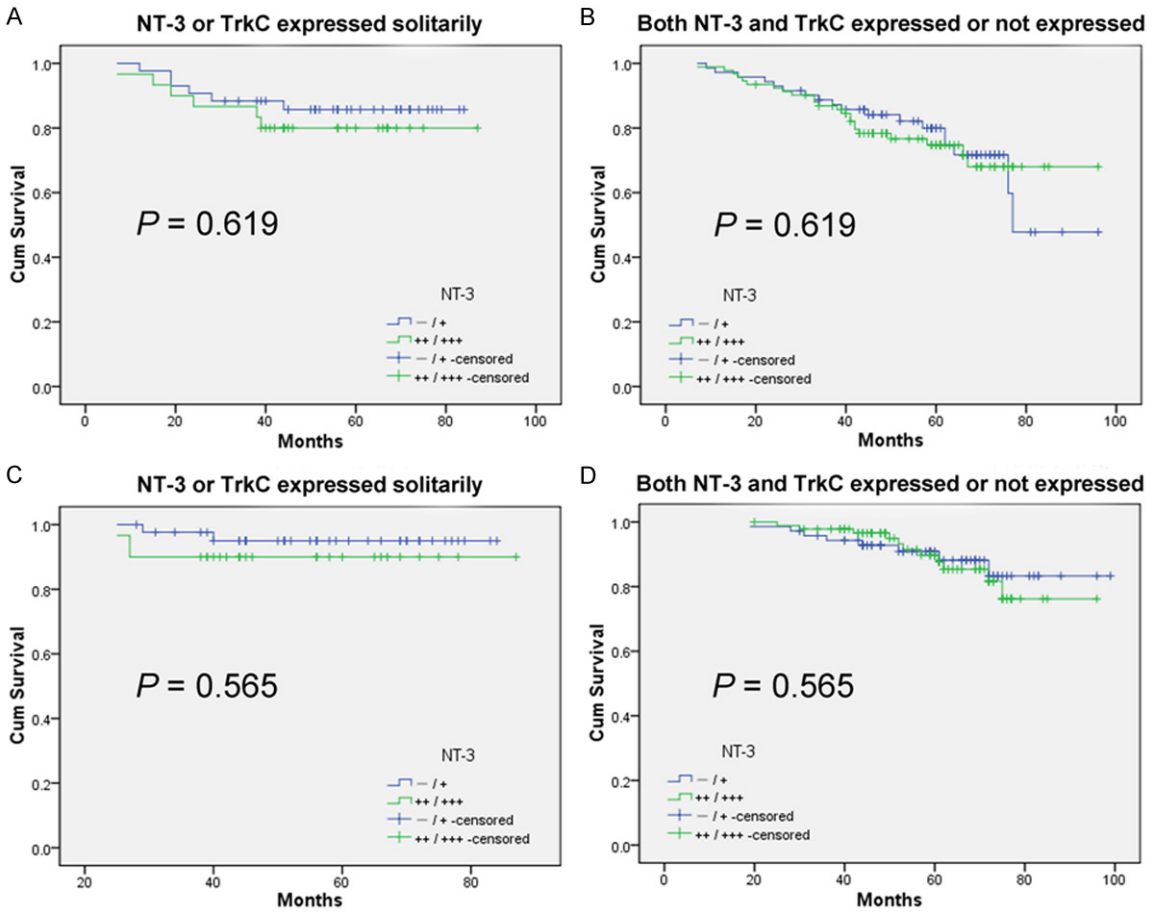
Cases No	TrkC (1×10^{-3})	Ratio (N/T)	Cum. Percent (%)
1	3.58	0.39	0.0
2	0.32	0.66	4.3
3	0.01	1.00	8.7
4	0.08	1.62	13.0
5	0.61	1.70	17.4
6	0.09	2.33	21.7
7	0.09	2.33	30.4
8	0.24	3.08	34.8
9	0.48	6.58	39.1
10	0.07	6.86	43.5
11	0.34	6.88	47.8
12	0.32	8.09	52.2
13	0.30	8.93	56.5
14	0.32	10.69	60.9
15	0.09	13.22	65.2
16	0.02	14.00	69.6
17	0.04	17.00	73.9
18	0.14	17.21	78.3
19	0.08	21.50	82.6
20	0.11	24.00	87.0
21	0.09	29.33	91.3
22	0.07	33.00	95.7
23	0.02	41.00	100.0

TrkC expression and its tumor/normal expression ratio measured by quantitative real time RT-PCR in 23 IDC samples. GAPDH expression was used as an internal control. Values obtained were divided by 1,000. The cumulative percentage of ratio (N/T) was also listed.

Supplementary Table 2. The association between NT-3 expression and clinicopathological characteristics in IDC

Characteristics	NT-3 expression		χ^2	P
	—/ +, No (%)	++ / +++, No (%)		
Age			1.032	0.597
< 40	8 (7.0)	13 (10.7)		
40-50	39 (34.2)	42 (34.4)		
> 50	67 (58.8)	67 (54.9)		
Pathological tumor size			2.977	0.226
≤2 cm	36 (31.6)	44 (36.1)		
>2 cm, ≤5 cm	70 (61.4)	75 (61.5)		
>5 cm	8 (7.0)	3 (2.5)		
Tumor grade			3.190	0.203
1	20 (17.5)	23 (18.9)		
2	53 (46.5)	68 (55.7)		
3	41 (36.0)	31 (25.4)		
Lymph node status			0.136	0.712
Negative	58 (50.9)	65 (53.3)		
Positive	56 (49.1)	57 (46.7)		
AJCC stage			1.355	0.508
I	26 (22.8)	21 (17.2)		
II	61 (53.5)	73 (59.8)		
III	27 (23.7)	28 (23.0)		
ER			0.779	0.378
Negative	57 (50.0)	54 (44.3)		
Positive	57 (50.0)	68 (55.7)		
PR			0.972	0.324
Negative	46 (40.4)	57 (46.7)		
Positive	68 (59.6)	65 (53.3)		
HER2			0.773	0.679
Negative	93 (81.6)	95 (77.9)		
Positive	14 (12.3)	16 (13.1)		
Equivocal	7 (6.1)	11 (9.0)		
Ki-67			0.815	0.367
≤ 14%	33 (28.9)	29 (23.8)		
> 14%	81 (71.1)	93 (76.2)		
Molecular subtypes			2.191	0.534
Luminal A	58 (50.9)	57 (46.7)		
Luminal B	16 (14.0)	18 (14.8)		
HER2	7 (6.1)	14 (11.5)		
Triple negative	33 (28.9)	33 (27.0)		
Chemotherapy			0.031	0.861
Yes	102 (89.5)	110 (90.2)		
No	12 (10.5)	12 (9.8)		
Radiotherapy			0.136	0.712
Yes	28 (24.6)	32 (26.7)		
No	86 (75.4)	88 (73.3)		
Endocrine therapy			0.281	0.596
Yes	60 (52.6)	60 (49.2)		
No	54 (47.4)	62 (50.8)		

Chi-square test is used. *P* values ≤ 0.05 are considered statistically significant.



Supplementary Figure 1. The survival analysis for invasive ductal carcinoma (IDC) patients with different NT-3 expression in a layered comparison based on the correlation of NT-3 and TrkC expression. Whether in the solitary expression of NT-3 or TrkC (A, C) or in the co-expression of NT-3 and TrkC (B, D), NT-3 expression didn't correlated with disease free survival (DFS) (A, B) and overall survival (OS) (C, D) ($P > 0.05$).