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Original Article

High expression of brain-derived neurotrophic factor in intrahepatic cholangiocarcinoma is associated with intraneural invasion and unfavorable prognosis

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Abstract: Aim: Brain-derived neurotrophic factor (BDNF) could promote the survival and differentiation of neural cells in peripheral and central nervous systems during development. Emerging evidences identified BDNF as an oncoprotein which could promotes progression and prognosis of tumors such as giloma, lung cancer and gastric cancer. We performed experiments to investigate the expression and clinical significance of BDNF in intrahepatic cholangiocarcinoma (IHCC). Materials and methods: The expression of BDNF and vascular endothelial growth factor (VEGF) was detected with immunohistochemistry in 96 patients with cholangiocarcinoma. The correlations between BDNF and the clinicopathologic factors were evaluated with Fisher test. The prognostic values of BDNF and VEGF were analyzed by the univariate analysis with Kaplan-Meier test and independent prognostic factor was identified by multivariate analysis with Cox-regression model. The effect of endogenous and exogenous BDNF on the invasion of IHCC cell line RBE was explored by transwell assay. Results: The percentage of high expression of BDNF was 35.96% (34/96). High expression of BDNF was significantly associated with positive intraneural invasion (P=0.012) and low overall survival rate (P=0.006). High expression of BDNF was identified as an independent prognostic factor in IHCC (P=0.032). With Matrigel transwell assay, we demonstrated that both endogenous and exogenous BDNF could promote the invasion of IHCC cells. Conclusions: High expression BDNF was identified as an independent risk in IHCC indicating poorer prognosis. Both endogenous and exogenous BDNF could promote the invasion of IHCC cells, indicating that BNDF may promote IHCC invasion in a paracrine or autocrine pathway.

Keywords: Brain-derived neurotrophic factor, intrahepatic cholangiocarcinoma, intraneural invasion, prognosis

Introduction

Biliary tract cancer is heterogeneous tumors that may arise anywhere from the epithelium of biliary tree, consisting of intrahepatic cholangiocarcinoma (IHCC), perihilar cholangiocarcinoma, extrahepatic cholangiocarcinoma and gallbladder cancer [1]. Most cholangiocarcinomas are adenocarcinomas while other rare histological subtypes are reported sporadically [2]. IHCC accounts for only less than 10% of all cholangiocarcinoma cases [3], but most patients with IHCC are diagnosed at an advanced stage because its clinical symptoms are very silent. The overall survival rate of IHCC remains very poor even after radical surgery, partially because of the poor response of IHCC to adjuvant therapy [4]. The patients with IHCC usually suffer early recurrence and lymphatic metastasis, leading to the unfavorable prognosis. There are some merging molecular targets of IHCC along with years of exploration, such as VEGF, epidermal growth factor receptor (EGFR) or human epidermal growth factor receptor-2 (HER2), but many of them are based on laboratory study without clinical experiments [5]. The need for more useful predictive or prognostic biomarkers is still urgent to push the treatment revolution of IHCC.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, mainly involved in the development and repair of nerve system [6]. BDNF regulate many cellular processes like cell survival and differentiation via binding with the tropomyosin-related receptor

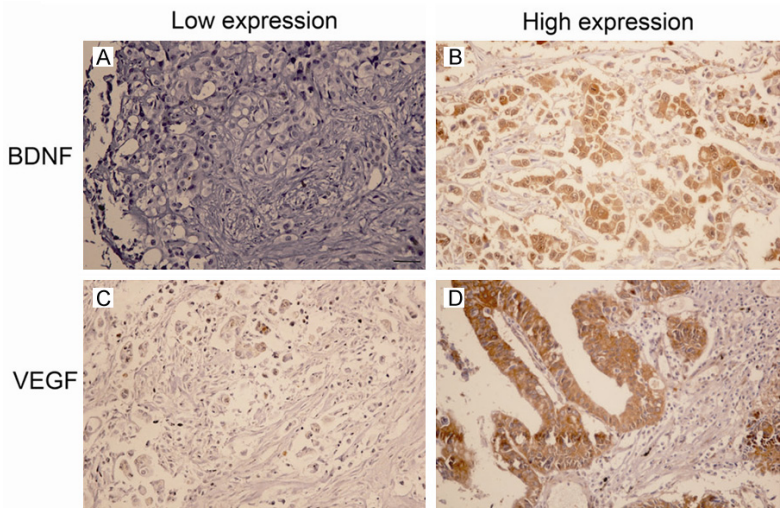

Figure 1. Images of representative immunohistochemical staining of BDNF and VEGF in intrahepatic cholangiocarcinoma. A and B. Representative images of BDNF low-expression and high-expression. Scale bar: 50 μm. C and D. Representative image of VEGF low-expression and high-expression.

kinase B (TrkB) receptor or the pan-NT receptor p75 (p75NTR) receptor. Besides the function in neuronal development, BDNF was demonstrated to be associated with the proliferation, differentiation, survival or apoptosis of neuronal and non-neuronal tumors by mediating the signaling pathway of RAS/MAPK or PI3K/AKT [7]. Moreover, ectopic expression or function of BDNF was demonstrated to promote cancer progression, response to chemotherapy or prognosis in a variety of cancers including glioblastoma, colorectal cancer, lung cancer, or hepatocellular carcinoma and so on [6, 8, 9]. However, the function of BDNF in IHCC is not explored partially because of the low incidence of IHCC and the difficulty to get the specimens for study.

In our experiment, we explored the expression and clinical significance of BDNF with VEGF, one known prognostic marker of IHCC, as a positive control. We further investigated the influence of BDNF on IHCC cell line invasion with Matrigel assay and concluded that high BDNF could lead to poorer prognosis via promoting the intraneural invasion of IHCC.

Materials and methods

Patients and follow-up

The study was approved and supervised by the Ethics Review Board of Qilu Hospital affiliated to Shandong University and Shanxian Central Hospital. All the 96 patients with IHCC underwent radical resection from 2002 to 2010, and the diagnosis was confirmed by the routine pathology. The patients were selected into our cohort following the criteria published before [10]: (1) available formalin-fixed tumor tissues, (2) available clinical follow-up data and complete medical records, (3) no history of previous anticancer therapy or post-operational adjuvant therapy. The tissue specimens were obtained from the Department of Pathology with prior consent of patients and the clinical data were abstracted from the patients' medical records. The pathologic tumor-node-metastasis (pTNM) stage was referring to the 7th staging classification of AJCC/UICC.

Immunohistochemistry (IHC) and evaluation

The protocol of IHC staining and score system was described before in detail [11]. The results of immunohistochemistry were evaluated independently by two senior pathologists unaware of the clinical data. Briefly, the final IHC score was defined by the product of score for percentage of stained cells multiplied by the score for staining intensity [12]. The staining intensity was identified as: score 0 for negative, score 1 for weak, score 2 for moderate and score 3 for strong. The score of percentage of positive staining cells was defined as: 1, <10% of cells were positive; 2, 10-25% of cells were positive; 3, 25-50% of cells were positive. ; 4, >50% of cells were positive. The cut-off scores of BDNF and VEGF were identified by the ROC curve according to previous studies [13, 14], which was 3.2 and 5.5, respectively.

Cell culture and transfection

The human IHCC cell line RBE was purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, USA) and 1% ampicil-

Table 1. Correlations between clinicopathologic factors and BDNF in patients with intrahepatic cholangiocarcinoma

Clinicopathologic parameters		n	BDNF		P*
			Low	High	
Age (years)	<65	70	46	24	0.811
	≥65	26	16	10	
Sex	Male	50	31	19	0.671
	Female	46	31	15	
Tumor size (cm)	<5 cm	31	19	12	0.655
	≥5 cm	65	43	22	
Differentiation	Good	27	18	9	0.571
	Moderate	47	32	15	
	Poor	22	12	10	
T stage	T1+2	71	48	23	0.336
	T3+4	25	14	11	
N stage	N0	67	47	20	0.086
	N1	29	15	14	
M stage	M0	92	61	31	0.126
	M1	4	1	3	
TNM stage	I+II	50	36	14	0.236
	III+IV	46	26	20	
HBV	Negative	85	53	32	0.318
	Positive	11	9	2	
Microvascular invasion	Negative	78	49	29	0.588
	Positive	18	13	5	
Cirrhosis	Negative	79	51	28	0.612
	Positive	17	11	6	
Intraneural invasion	Negative	85	59	26	0.007
	Positive	11	3	8	
VEGF	Low	47	33	14	0.291
	High	49	29	20	

*Means calculated by Fisher test. Abbreviations: BDNF = brain-derived neurotrophic factor, vascular endothelial growth factor = VEGF.

lin/streptomycin (HyClone, USA) in 5% CO_2 resuscitation.

The antibodies of BDNF and β-actin, and the siRNA of BDNF were purchased from the Santa Cruz Biotechnology (Santa Cruz, CA, USA). The human recombinant BDNF was purchased from PeproTech Company. All other agents were purchased from the Sigma-Aldrich Cooperation if there is no special instruction. The transfection of siRNA and scrambled RNA into RBE was realized by the lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manual.

Western blotting

The verification of knockdown results was realized by Western blotting according to previous study [15]. The cell after transfection were lysed with RIPA lysis buffer and scraped on ice. The precipitate was discarded and supernatant was mixed with 2× sample buffer following centrifuge at 10000× g for 30 minutes. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to electrophorese the proteins. After transferring the proteins to nitrocellulose membrane (PALL Corporation, New York, USA), primary antibody of BDNF at dilution of 1:100 was used to incubate the membrane overnight at 4°C. The HRP-conjugated secondary antibody (Beyotime Company, Shanghai, China) was used to incubate the membrane for 2 hours after rising with PBS and proteins were visualized by enhanced chemi-luminescence assay (ECL).

Matrigel invasion assay

Invasion of RBE was detected with transwell assay 8-μm-pore Matrigel-coated transwells (BD Biosciences, NJ, USA) as reported before [16]. RBE cells were passaged into the transwell chambers and starved in serum-free medium for 6 hours. The upper chamber was incubated with 1%-serum-containing medium, and the lower chamber was incubated with 10% serum as chemo-attractant. The cells were incubated for 24 hours for invasion. After fixed by 4% paraformaldehyde and stained with 0.05% gentian violet, the cell number of the lower surface was counted from 8 random visual fields under microscope.

Statistical analysis

The software SPSS 22.0 software was used to analyze all the data if there is no special instruction. Fisher test was applied to analyze the correlation between BDNF and clinicopathologic features. Kaplan-Meier method was used to display the survival curves and log-rank test was used to compare the statistical difference of subgroups in survival analysis. The Cox regression hazards model was used to identify

Table 2. Univariate analysis of clinicopathologic parameters in patients with intrahepatic cholangiocarcinoma

Clinicopathologic parameters		5-year survival %	P*
Age (years)	<65	42.3	0.940
	≥65	59.2	
Sex	Male	43.0	0.184
	Female	46.5	
Tumor size (cm)	<5 cm	60.7	0.082
	≥5 cm	36.3	
Differentiation	Good	42.0	0.446
	Moderate	39.6	
	Poor	55.5	
T stage	T1+2	51.2	0.362
	T3+4	28.9	
N stage	N0	55.5	P<0.001
	N1	13.0	
M stage	M0	45.5	0.256
	M1	25.0	
TNM stage	I+II	61.0	0.008
	III+IV	27.6	
HBV	Negative	44.0	0.821
	Positive	57.3	
Microvascular invasion	Negative	45.1	0.633
	Positive	45.8	
Cirrhosis	Negative	42.7	0.889
	Positive	57.8	
Intraneural invasion	Negative	44.3	0.743
	Positive	46.2	
BDNF	Low	56.4	0.006
	High	25.4	
VEGF	Low	59.5	0.022
	High	20.4	

*Means calculated by log-rank test, Abbreviations: BDNF = brain-derived neurotrophic factor, vascular endothelial growth factor = VEGF.

the independent prognostic factors. Student's t test was used to compare different groups of Matrigel assay. *P*-values <0.05 was considered to be significant.

Results

The expression of BDNF and VEGF in IHCC tissue

Immunohistochemistry was used to detect the expression and intracellular localization of BDNF (**Figure 1A** and **1B**). As a well-acknowledged biomarker in IHCC, the expression of VEGF was also investigated as a positive control and verification of our cohort (**Figure 1C** and **1D**). In our experiment, both BDNF and VEGF were mainly observed in the cell cytoplasm. According to the standard detailed in *material and methods*, the cohort was divided into low-expression and high-expression subgroup depending on the score of BDNF and VEGF. The percentages of low-expression and high-expression subgroup of BDNF were 35.96% (34/96) and 64.58% (62/96).

Correlation between BDNF and clinicopathological factors

The correlations between BDNF expression and clinicopathological factors were analyzed by Fisher test (**Table 1**). In our experiment, the expression of BDNF was significantly associated with intraneural invasion (P=0.007). Patients with higher BDNF expression seemed to be more vulnerable to intraneural invasion. Moreover, the BDNG high expression appeared to be related to positive lymphatic invasion and distant metastasis, but the tendency was no statistically significant (P=0.086 and 0.126, respectively).

Prognostic significance of BDNF in IHCC

The correlation between BDNF expression, clinicopathological factors and the 5-year survival rates was evaluated by the univariate analysis by Kaplan-Meier test to evaluate the prognostic significance (**Table 2**). In our test, the expression of BDNF and VEGF was both significantly associated with the prognosis of patients with IHCC. The patients with high expression of BDNF and VEGF had lower survival rate compared with those with low expression of BDNF and VEGF (**Figure 2A** and **2B**). Additionally, advanced N stage (P<0.001) and TNM stage (P=0.008) also predict the poorer prognosis of IHCC patients. Patients with larger tumor size seemed to have a lower survival rate compared with those with small tumor size, with a statistically insignificant tendency (P=0.082).

With multivariate analysis, we identified independent prognostic factors of IHCC (**Table 3**). We expanded the criteria to P<0.1 and enrolled

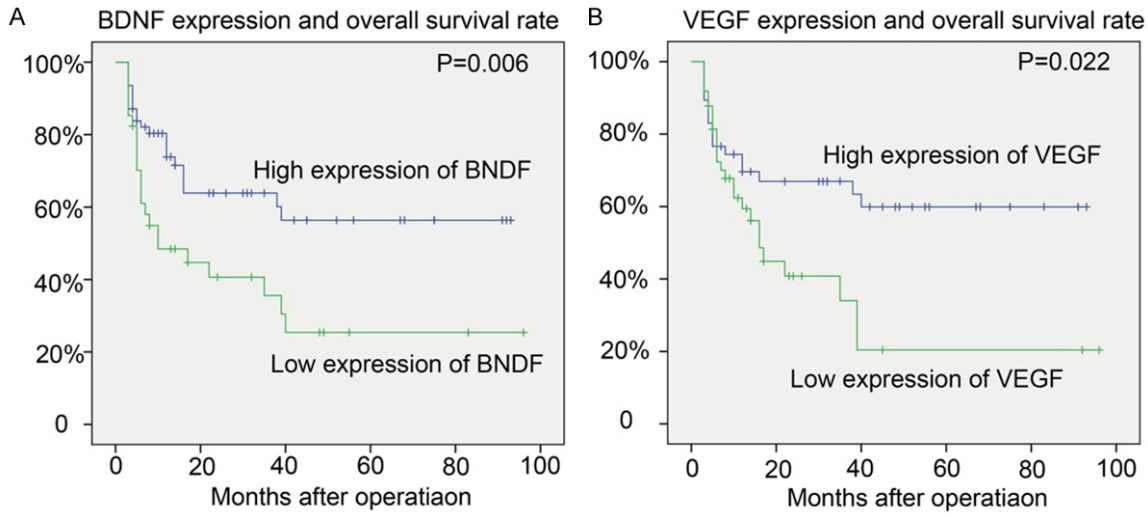

Figure 2. The correlation between BDNF, VEGF and survival rate in patients with intrahepatic cholangiocarcinoma. A. The overall survival curves of BDNF low-expression and high-expression. The patients with high BDNF expression had a poorer prognosis compared with those with low BDNF expression (P=0.006). B. The overall survival curves of VEGF low-expression and high-expression. The patients with high VEGF expression had a poorer prognosis compared with those with low VEGF expression (P=0.022).

Table 3. Multivariate analysis of clinicopathologic parameters in patients with intrahepatic cholangiocarcinoma

Factor	Category	HR	95% CI	P*
Tumor size	<5 cm	1.00		0.234
	≥5 cm	1.61	0.74-3.50	
N stage	N0	1.00		0.031
	N1	2.14	1.07-4.26	
BDNF	Low	1.00		0.032
	High	2.00	1.06-3.76	
VEGF	Low	1.00		0.734
	High	1.14	0.54-2.34	

*Means calculated by Cox-regression model. Abbreviations: BDNF = brain-derived neurotrophic factor, vascular endothelial growth factor = VEGF. HR = hazard ratio, 95% CI = 95% confidence interval.

tumor size, N stage, BDNF and VEGF into the Cox-regression model. BDNF (P=0.032, HR= 2.00, 95% CI=1.06-3.76) and N stage (P=0.031, HR=2.14, 95% CI=1.07-4.26) was confirmed as the independent prognostic factor of IHCC. High expression of BDNF and advanced N stage could predict the unfavorable prognosis of patients with IHCC.

Endogenous and exogenous BDNF could promote the invasion of IHCC cells

We observed that high expression of BDNF was significantly associated with intraneural invasion, indicating that BDNF could facilitate the ability of invasion of IHCC cells. We performed transwell assay to detect the invasion of IHCC cell RBE. The BDNF expression of RBE was first knocked down with transfection of BDNF siRNA and the knocking-down results were verified by Western blotting (**Figure 3A**). After BDNF knockdown, the invasion of RBE cells was detected. The invasion of cells with BDNF knockdown decreased significantly compared with cells with scrambled siRNA as control (**Figure 3B**). The effect of exogenous BDNF on RBE cells was also investigated with recombinant human BDNF. Different dose of BNDF from 0 to 500 ng/ml was used to incubate RBE cells for 24 hours for invasion. The results suggested that exogenous BDNF could promote the invasion of IHCC cell line in a dose-effect manner (**Figure 3C**).

Discussion

There are more and more drug targets identified in IHCC, predicting potential targeted drug and more treatment choice. VEGF, EGFR and Her2 were all demonstrated to be related with the progression and prognosis of IHCC as the potential drug targets, and there corresponding inhibitors or antibodies were proved to inhibit cancer cell progression with experiments *in vivo* and *in vitro* [17]. Some promising inhibitors are in different stages of pre-clinical or clinical studies, such as gefitinib, erlotinib and NVP-AEE788 [5]. The finding of more effective bio-

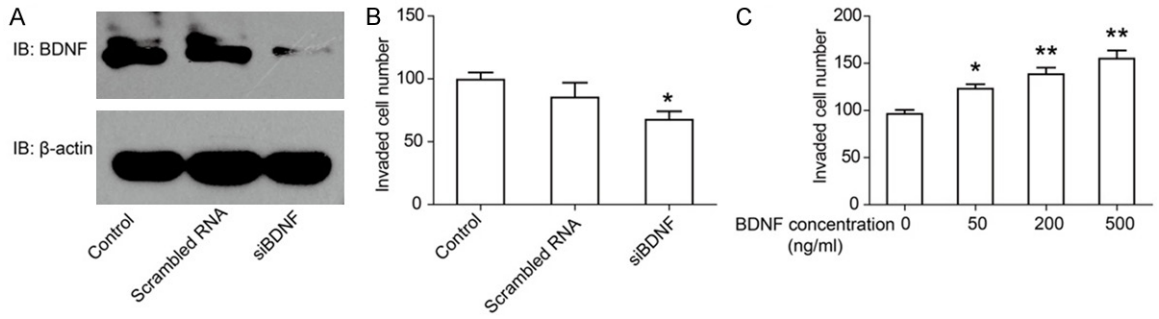

Figure 3. Endogenous and exogenous BDNF could promote IHCC invasion. A. Successful knockdown of BDNF in RBE cells was verified by Western blotting. B. Endogenous BDNF could promote the invasion of RBE cells. RBE cell invasion was detected by the transwell assay 48 hours after the transfection of BDNF siRNA or scrambled siRNA. *means $P<0.05$. C. Exogenous BDNF could promote IHCC invasion. Human recombinant BDNF at concentration at 0, 50, 200, 500 ng/ml was used to incubate RBE cells for 24 hours and the invasion was detected by the transwell assay. *means $P<0.05$, and **means $P<0.01$.

markers could accelerate the exploration of drug target and more drugs to IHCC. Since BDNF played an important role in neural development and repair, the studies on BDNF agonist were much more than those on BDNF antagonist. The exploration on BDNF inhibitor was rare and inhibiting BDNF systemically may lead to severe nervous problems like depression. So the study of delivering the inhibitor to BDNF or its downstream signaling molecular to the tumor preciously would be more promising to the treatment of IHCC.

Intraneural invasion is a major invasion pathway of IHCC and partially resulted in the high recurrence rate. Several proteins like nerve growth factor (NGF), neural cell adhesion molecule (NCAM), matrix metalloproteinase (MMP), and acetylcholine (Ach) have been proved to be related to the process of intraneural invasion. Some studies reported that survival rate of patients with chlangiocarcinoma with intraneural invasion is remarkably lower than those without intraneural invasion [18]. In our study, we did not observe a significant correlation between IHCC prognosis and intraneural invasion, which may be resulted from the limited sample size. It is intriguing that we proved that BDNF was related to intraneural invasion, which means that BDNF detection could stratify the high-risk patients more preciously and may help find potential drug target for IHCC treatment.

In our study, we demonstrated that BDNF overexpression could lead to aggressive progression and poorer prognosis, but the underlying mechanism is still in the mist. There are two well-known receptors of BDNF, which are the tropomyosin-related receptor kinase B (TrkB) and the pan-NT receptor p75 (p75NTR). The former one has high affinity and specificity to BDNF and the latter one has lower affinity [19]. The interaction with TrkB could result in the activation of downstream signaling pathways such as RAS/MAPK and PI3K/AKT, which was involved in many processes of tumor progression like proliferation, apoptosis or invasion. In our experiment, we demonstrated that high BDNF expression in IHCC lead to easier intraneural invasion and poorer prognosis, suggesting the intracellular BDNF facilitates the progression of IHCC. We further proved that both endogenous and exogenous BDNF could promote the invasion of IHCC cells. Considering that BDNF is a kind of secretory protein, IHCC cells with high BDNF are very likely to have more aggressive invasion via the ectopic activation of BDNF signaling pathway in a paracrine or autocrine pathway. This suggestion certainly needs more basic experiment to demonstrate.

In summary, we detected the expression of BDNF in 96 cases of IHCC and demonstrated that BDNF expression was significantly associated with lower 5-year survival rate. High expression of BDNF was identified as an independent prognostic factor of IHCC. With experiments *in vitro*, we demonstrated that both endogenous and exogenous BDNF could promote the invasion of IHCC cells, indicating that BNDF may promote IHCC invasion in a paracrine or autocrine pathway.

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Disclosure of conflict of interest

None.

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