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Brain-derived neurotrophic factor/ tropomyosin-related kinase B pathway in gastric cancer

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Background: Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family that is known to activate the high-affinity tropomyosin-related receptor kinase B (TrkB). This study aimed to clarify the clinical and biological significance of the BDNF/TrkB pathway in gastric cancer.

Methods: We analysed BDNF and TrkB expression in gastric cancer samples by real-time reverse transcription PCR and immunohistochemistry. To investigate the biological role of BDNF/TrkB axis, recombinant human BDNF (rhBDNF) and the Trk antagonist K252a were used for *in vitro* and *in vivo* analysis.

Results: The BDNF expression at the invasive front of primary tumours was significantly elevated compared with that in the tumour core and adjacent normal mucosa. Increased BDNF expression at the invasive front was significantly correlated with factors reflecting disease progression, and poor prognosis. Increased co-expression of the BDNF/TrkB axis was significantly correlated with poor prognosis. Gastric cancer cells expressed BDNF, and administration of rhBDNF promoted proliferation, migration, invasion, and inhibition of anoikis. These effects were generally inhibited by K252a. In an *in vivo* assay, BDNF(+)/TrkB(+) gastric cancer cells injected into nude mice established peritoneal dissemination, whereas K252a inhibited tumour growth.

Conclusion: The BDNF/TrkB pathway might be deeply involved in gastric cancer disease progression.

Despite a decline in its incidence, gastric cancer remains the second leading cause of cancer death worldwide (Ferlay *et al*, 2010). At the time of initial diagnosis, many cases have already reached an advanced stage where tumour cell spreading has occurred. For better diagnosis and treatment of gastric cancer, identification of the pathways responsible for disease development and progression is critical.

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family and is known to activate the high-affinity tropomyosin-related receptor kinase B (TrkB) receptor. The BDNF/TrkB pathway has a key role in the development of the nervous system and participates in regulating neuronal survival,

differentiation, and activity through synaptic modulation (Lewin and Barde, 1996; Segal and Greenberg, 1996). Douma *et al* (2004) demonstrated that TrkB generates a specific and potent pro-survival signal that renders epithelial cells resistant to anoikis, whereupon they acquire potent tumorigenic, invasive, and metastatic capacities. Several studies indicated that BDNF and/or its receptor, TrkB, might be involved in cancer growth and metastasis in several malignant tissues, such as neuroblastoma (Aoyama *et al*, 2001), pancreatic ductal carcinoma (Miknyoczki *et al*, 1999), prostate cancer (Montano and Djamgoz, 2004), lung cancer (Ricci *et al*, 2001), and hepatocellular carcinoma (Yang *et al*, 2005). The TrkB activation by BDNF was shown to enhance the

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proliferation and survival of transitional cell carcinoma cell lines, whereas a TrkB antagonist suppressed their migration and invasion (Huang *et al*, 2010a, b). Previously, we used RNA interference in colorectal cancer (CRC) cells to demonstrate that high levels of TrkB transcript expression is associated with poor prognosis in CRC patients and enhanced malignant potential in terms of proliferation, migration, invasion, and anoikis inhibition (Fujikawa *et al*, 2012). In addition, in gastric cancer patients, we revealed that high TrkB transcript expression is an independent prognostic marker (Tanaka *et al*, 2009).

Although a growing number of studies demonstrated the function of the BDNF/TrkB axis in experimental systems, to date no reports have shown a biological function of the BDNF/TrkB pathway in gastric cancer cell lines or a correlation between BDNF/TrkB expression and clinicopathological variables, including prognosis. The aim of this study was therefore to determine the relationship between BDNF/TrkB expression determined by immunohistochemical analysis and clinicopathological findings in gastric cancer patients and to reveal the function of the BDNF/TrkB axis in gastric cancer *in vitro* and *in vivo*.

MATERIALS AND METHODS

Patients and sample collection. A total of 150 patients (119 men and 31 women) of our study, accompanied with the availability of cancer fresh frozen samples with complete clinical data, the quality of isolated RNA for real-time PCR, and preserved high-quality paraffin block of resected primary cancer specimen, were consecutive patients who underwent surgery for gastric cancer from January 2000 to November 2009 at Mie University Hospital, Japan. Further information is provided in the Supplementary Data. The study design was approved by the Mie University institutional review board, and written informed consent was obtained from all patients.

Immunohistochemical analysis. Immunohistochemical analysis was performed as described previously (Fujikawa *et al*, 2012). A primary rabbit polyclonal antibody against BDNF (H-117, 1:350; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and primary mouse monoclonal antibody against TrkB (1:100; R&D Systems, Foster City, CA, USA) were used in a labelled streptavidin-biotin method (LAS2 kit/HRP; Dako Cytomation, Carpinteria, CA, USA). Negative controls were also run simultaneously.

Evaluation of the degree of antibody reactivity. The immunoreactivity of BDNF and TrkB in each primary gastric cancer was separately scored for the tumour invasion front (usually <0.5 mm broad in tumour periphery) and the core of the tumour (centre of the tumour in general, excluding the invasion front and necrotic areas), as previously described (Alpizar-Alpizar *et al*, 2011), by scanning the whole section at medium ($\times 50$) and high magnification ($\times 200$) as: no staining, 0; weak staining, light yellow, 1; moderate staining, yellowish brown, 2; and strong staining, brown, 3. The slides were evaluated three times by three independent investigators (YO, KT and SS) who were blinded to the nature of the specimens and antibodies used. To compare the expression of BDNF and/or TrkB with clinical and pathological parameters, samples were grouped in high (staining intensity > median immunohistochemical score = 1) and low-expression groups (staining intensity \leq median immunohistochemical score = 1) as described previously (Kahlert *et al*, 2011). In addition, BDNF expression was further analysed by dichotomised as upregulation group (staining intensity: invasive front > core site) and no change or downregulated group (staining intensity: invasive front \leq core site).

Total RNA extraction, cDNA synthesis, and semi-quantitative RT-PCR. Tumour specimens and gastric cancer cell lines were homogenised with a Mixer Mill MM 300 homogeniser (Qiagen, Chatsworth, CA, USA). Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesised from 5.0 mg total RNA with a random hexamer primer and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Semi-quantitative PCR (qPCR) analysis was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The relative abundance of target transcripts that were measured using TaqMan probes for *BDNF* (Assay ID, Hs00380947_m1, TaqMan Gene Expression Assays; Applied Biosystems) and *Ntrk2* (Assay ID, Hs00178811_m1; Applied Biosystems) was normalised to the expression level of β -actin (Assay ID, Hs01060665_g1; Applied Biosystems). TaqMan probes for *BDNF* corresponded to exon 1-2 of *BDNF* transcript variant 5 (NCBI reference sequence: NM_170733.3). Amplified products were separated electrophoretically, visualised, and photographed under UV light after ethidium bromide staining, and quantified by CA Analyser version 2.0 (ATTO Corporation, Tokyo, Japan). Densitometry analyses were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Real-time quantitative RT-PCR and relative expression level of BDNF. Quantitative PCR analysis was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems). The relative abundance of target transcripts that were measured using TaqMan probes for *BDNF* (Assay ID, Hs00380947_m1; Applied Biosystems) was normalised to the expression level of *GAPDH* (Assay ID, Hs02758991_g1; Applied Biosystems) and evaluated using Applied Biosystems StepOne Software v2.1. The relative BDNF gene-expression level was determined by the standard curve method. Further information is provided in the Supplementary Data.

Cell lines. Human gastric cancer cell lines MKN7 (intestinal type), MKN74 (intestinal type), MKN45 (diffuse type), KATO III (diffuse type) and NUGC3 (diffuse type) were obtained from the Cell Resource Center for Biomedical Research, Tohoku University. These cell lines have been tested and authenticated. These cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 IU ml⁻¹ penicillin, and 100 lg ml⁻¹ streptomycin at 37 °C and 5% CO₂.

Western blot analysis. Western blotting was performed as described previously (Toiyama *et al*, 2011). Immobilon membranes (Millipore, Billerica, MA, USA) were incubated with the respective anti-human primary antibody at the recommended dilution (anti-BDNF, TrkB, p-AKT, p-ERK, E-cadherin, β -actin (Santa Cruz Biotechnology)).

Pharmaceutical reagents. Recombinant human BDNF (rhBDNF) was purchased from PeproTech (Rocky Hill, NJ, USA) and prepared according to the manufacturer's instructions. K252a was purchased from (Calbiochem, San Diego, CA, USA) and stored at -20 °C before use *in vitro*. K252a was dissolved in PBS before animal injection.

Proliferation, cell cycle, invasion, migration, and anoikis assays. In order to assess the effect of BDNF on proliferation, invasion, and migration, 100 ng ml⁻¹ rhBDNF was used. To investigate the relationship of BDNF and TrkB, we used 50 nM Trk antagonist K252a. Effects of rhBDNF on GC cell lines were compared with untreated cells, cells treated with K252a, or with cells pre-treated with K252a for 2 h followed by rhBDNF. Further information is provided in the Supplementary Data.

In vivo studies. Male nude mice (BALB/c) at 8 weeks of age were obtained from Japan SLC. The treatment protocol followed the guidelines for animal experimentation adopted by Mie University, and meets the standards required by the UKCCCR guidelines (Workman *et al*, 2010). The experimental protocols were reviewed and approved by the Animal Care and Use Committee at the Mie University Graduate School of Medicine. NUGC cells (2×10^6 cells per 1 ml PBS) were injected into mice intraperitoneally to generate peritoneal metastasis of gastric cancer. To examine the effect of K252a on the peritoneal dissemination potential of gastric cancer

cells, 1 ml dissolved K252a ($500 \mu\text{g kg}^{-1}$) or 1 ml PBS (control) was injected intraperitoneally 14 days after carcinoma cell injection. Mice were killed 35 days after the injection of carcinoma cells, and intraperitoneal dissemination was evaluated ($n = 5$).

Statistical analysis. Statistical analysis was performed using StatView software (version 5; Abacus Concepts, Inc., Berkeley, CA, USA). Results are expressed as means \pm s.e. Differences between groups were estimated by the Pearson's χ^2 -test, repeated-measures analysis of variance analysis, or unpaired

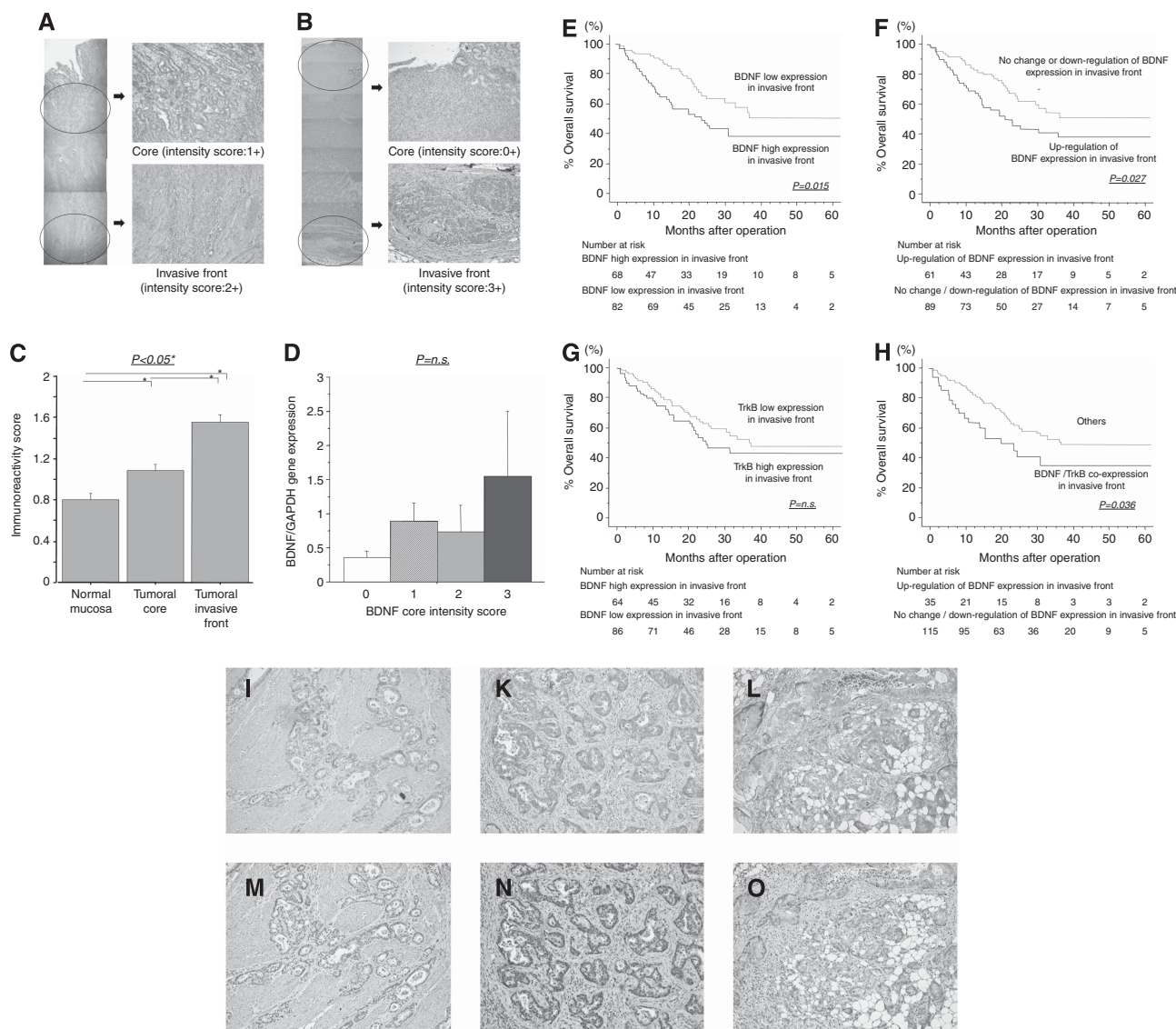


Figure 1. (A, B) Representative example of upregulated immunohistochemical BDNF staining in the tumour invasive front of primary gastric cancer compared with that in the tumour core. (A, B: original magnification: $\times 40$ and $\times 100$, respectively). In these representative images, BDNF immunoreactivity in each primary gastric cancer was scored as: no staining, 0; weak staining, 1; moderate staining, 2; and strong staining, 3. (C) Immunohistochemical intensity scoring in primary gastric cancer and adjacent normal mucosa. BDNF expression in cancerous tissues was significantly higher than that in adjacent normal mucosa ($P < 0.05$). In addition, BDNF expression at the tumour invasive front was significantly higher than that in the tumour core ($P < 0.05$). (D) Quantitative real-time PCR analyses of BDNF gene expression relative to GAPDH in tumour core tissue specimens. The BDNF gene expression had a gradient elevation pattern similar to the increased immunohistochemical intensity, although they had no significant differences. (E–H) Kaplan–Meier survival curves of gastric cancer patients according to BDNF expression at the tumour invasive front based on immunohistochemical analysis. (E) The BDNF expression change comparing tumour invasive front and core (F) The TrkB expression in the tumour invasive front (G), and BDNF/TrkB co-expression in the tumour invasive front (H). (I–N) Representative example of positive immunohistochemical staining of BDNF and TrkB in a primary (I: BDNF, L: TrkB, original magnification $\times 100$) and disseminated lesion of the lymph node (J: BDNF, M: TrkB, original magnification $\times 100$) and peritoneum (K: BDNF, N: TrkB, original magnification, $\times 100$) from the same gastric cancer patient. BDNF and TrkB were also expressed in disseminated cancer cells of the lymph node and peritoneum in the same pattern as that observed in primary lesions of gastric cancer.

Student's *t*-test. Actuarial survival curves were obtained using the Kaplan–Meier method, and comparisons were made using log-rank tests. Two-sided *P*-values <0.05 were considered to be statistically significant.

RESULTS

Brain-derived neurotrophic factor overexpression at the invasive front of gastric cancer. The BDNF immunohistochemical studies revealed that the BDNF protein was mainly expressed in the cytoplasm of gastric cancer cells. Moreover, many cases had BDNF expression that was upregulated at the invasive front of primary gastric cancer compared with the corresponding tumour core (Figure 1A and B).

In clinical samples obtained from patients with gastric cancer, immunohistochemical analysis revealed that BDNF expression in cancerous tissues was significantly higher than that in adjacent normal mucosa (tumour-invasive front: 1.55 ± 0.85 ; tumour core: 1.09 ± 0.77 ; adjacent normal mucosa: 0.81 ± 0.72 , $P < 0.05$, respectively). In addition, BDNF expression at the tumour invasive front was significantly higher than that in the tumour core (Figure 1C). However, because evaluation by immunohistochemistry alone may be insufficient to determine whether BDNF is truly expressed, we evaluated BDNF expression by real-time quantitative RT-PCR. Expression values (relative mRNA levels) of BDNF were expressed as ratios between the gene of interest (BDNF) and an internal reference gene (GAPDH), which provided a normalisation factor for the amount of RNA. Relative BDNF gene expression was correlated with the BDNF expression score of the tumour core site in gastric cancer patients (Figure 1D).

Clinical significance of BDNF overexpression at the invasive front of gastric cancer. Table 1 shows the relationship between BDNF expression at the tumour invasive front and clinicopathological variables in tumour specimens from gastric cancer patients. Elevated BDNF expression was significantly associated with younger age (<69), vessel involvement, lymph node metastasis, and peritoneal dissemination. Patients with elevated BDNF expression at the tumour invasive front also had significantly poorer prognoses than those in the low-expression group (log-rank test, $P = 0.015$) (Figure 1E). In addition, patients with BDNF upregulation at the tumour invasive front compared with the tumour core had significantly poorer prognoses than those with equal or downregulated BDNF expression (log-rank test, $P = 0.027$) (Figure 1F).

Brain-derived neurotrophic factor/TrkB co-expression was significantly correlated with poor prognosis in gastric cancer. Immunohistochemical studies revealed that the TrkB protein was mainly expressed in the nucleus and cytoplasm of gastric cancer cells. Although TrkB expression at the tumour invasive front did not show a significant correlation with prognosis, the BDNF/TrkB co-expression group, where both BDNF and TrkB have high expression above cutoff levels, showed significantly worse prognoses than the other groups (log-rank test, $P = 0.036$) (Figure 1G and H). Table 2 shows the relationship between TrkB or BDNF/TrkB expression at the tumour invasive front and clinicopathological variables in gastric cancer patients. Besides the significant correlation with histological intestinal type, elevated TrkB expression had a tendency to be correlated with lymph node metastasis. The BDNF/TrkB co-expression was significantly associated with histological intestinal type and lymph node metastasis.

Immunohistochemical staining of BDNF and TrkB in gastric cancer tissues, lymph node metastasis, and peritoneal carcinomatosis. Immunohistochemical analysis revealed that BDNF and TrkB proteins were highly expressed in primary gastric cancer cells

Table 1. Clinicopathological Variables and BDNF expression in invasive front in 150 gastric cancer patients

Variable	n	BDNF expression		P
		High (n = 68)	Low (n = 82)	
Gender				
Male	119	52	67	0.431 ^b
Female	31	16	15	
Age (years)				
<69 (median)	71	42	37	0.042 ^{a,b}
≥69	79	26	45	
Tumour size				
≥5.5 cm (median)	76	37	39	0.404
<5.5 cm	74	31	43	
Histological type				
Intestinal type	75	32	43	0.512 ^b
Diffuse type	75	36	39	
Pathological T category				
pT1/2	52	19	33	0.115 ^b
pT3/4	98	49	49	
Vessel involvement				
+	119	61	58	0.004 ^{a,b}
–	31	7	24	
Lymphatic vessel involvement				
+	138	65	73	0.140 ^b
–	12	3	9	
Lymph node metastasis				
N0	45	13	32	0.008 ^{a,b}
N1	105	55	50	
Liver metastasis				
H0	138	63	75	0.790 ^b
H1	12	4	8	
Peritoneal carcinomatosis				
P0	123	51	72	0.042 ^{a,b}
P1	27	17	10	
Stage classification				
Stage I–II	48	17	31	0.094 ^b
Stage III–IV	102	51	51	

^a $P < 0.05$.

^bPearson's χ^2 -test.

and disseminated cancer cells of the lymph node and peritoneum in the same pattern as that observed in the primary lesions (Figure 1I–N).

BDNF promotes cell proliferation in gastric cancer cells expressing TrkB *in vitro*. Western blotting revealed that BDNF was detected in all gastric cancer cells tested, although semi-quantitative RT-PCR analysis demonstrated that BDNF transcript variant 5 was detected only in MKN7, MKN45, and NUGC3 cells (Figure 2A). Semi-quantitative RT-PCR and western blotting revealed that TrkB was expressed in all gastric cancer cells tested,

Table 2. Clinicopathological variables and BDNF/TrkB expression in 150 gastric cancer patients

Variable	n	TrkB expression		P	BDNF/TrkB axis		P
		High (n = 64)	Low (n = 86)		Co-expression (n = 35)	Others (n = 115)	
Gender							
Male	119	54	65	188 ^b	29	90	0.557 ^b
Female	31	10	21		6	25	
Age (y)							
< 69 (median)	71	30	41	0.923 ^b	12	59	0.078 ^b
≥ 69	79	34	45		23	56	
Tumour size							
≥ 5.5 cm (median)	76	34	42	0.603 ^b	20	56	0.382 ^b
< 5.5 cm	74	30	44		15	59	
Histological type							
Intestinal type	75	39	36	0.021 ^{a,b}	24	51	0.012 ^{a,b}
Diffuse type	75	25	50		11	64	
Pathological T category							
pT1/2	52	18	34	0.146 ^b	9	43	0.204 ^b
pT3/4	98	46	52		26	72	
Venous invasion							
+	119	51	68	0.926 ^b	30	89	0.287 ^b
-	31	13	18		5	26	
Lymphatic invasion							
+	138	61	77	0.197 ^b	33	105	0.569 ^b
-	12	3	9		2	10	
Lymph node metastasis							
N0	45	14	31	0.061 ^b	5	40	0.021 ^{a,b}
N1	105	50	55		30	75	
Liver metastasis							
H0	138	58	80	0.592 ^b	33	105	0.569 ^b
H1	12	6	6		2	10	
Peritoneal carcinomatosis							
P0	123	50	73	0.287 ^b	27	96	0.393 ^b
P1	27	14	13		8	19	
Stage classification							
Stage I-II	48	17	31	0.218 ^b	8	40	0.185 ^b
Stage III-IV	102	47	55		27	75	

^aP < 0.05.
^bPearson's χ^2 -test.

although MKN45 cells showed low TrkB protein expression (Figure 2B). Among these cell lines, MKN7, MKN74, and NUGC3 cells were selected for use in *in vitro* analysis because of their high expression levels of both BDNF and TrkB. The rhBDNF exposure upregulated the expression of p-AKT and p-ERK in these cell lines, and these expression changes could be inhibited by treating cells with the Trk inhibitor K252a for 20 min after rhBDNF exposure. In these gastric cancer cells, E-cadherin impairment was detectable 24 h after BDNF treatment, and these phenomena could also be blocked by K252a pre-treatment (Figure 2C).

In addition, we investigated the proliferative potential of the gastric cancer cell lines MKN7, MKN74, and NUGC3 treated with rhBDNF (100 ng ml⁻¹), K252a (50 nM), or K252a followed by rhBDNF. At 72 h after treatment, there was a significant increase in proliferation of rhBDNF-treated gastric cancer cells compared with that of untreated cells, and K252a treatment followed by rhBDNF inhibited the effect of rhBDNF in these cell lines. In addition, K252a treatment of MKN7 and NUGC3 cells without rhBDNF significantly reduced cell growth compared with that of untreated cells (Figure 3A).

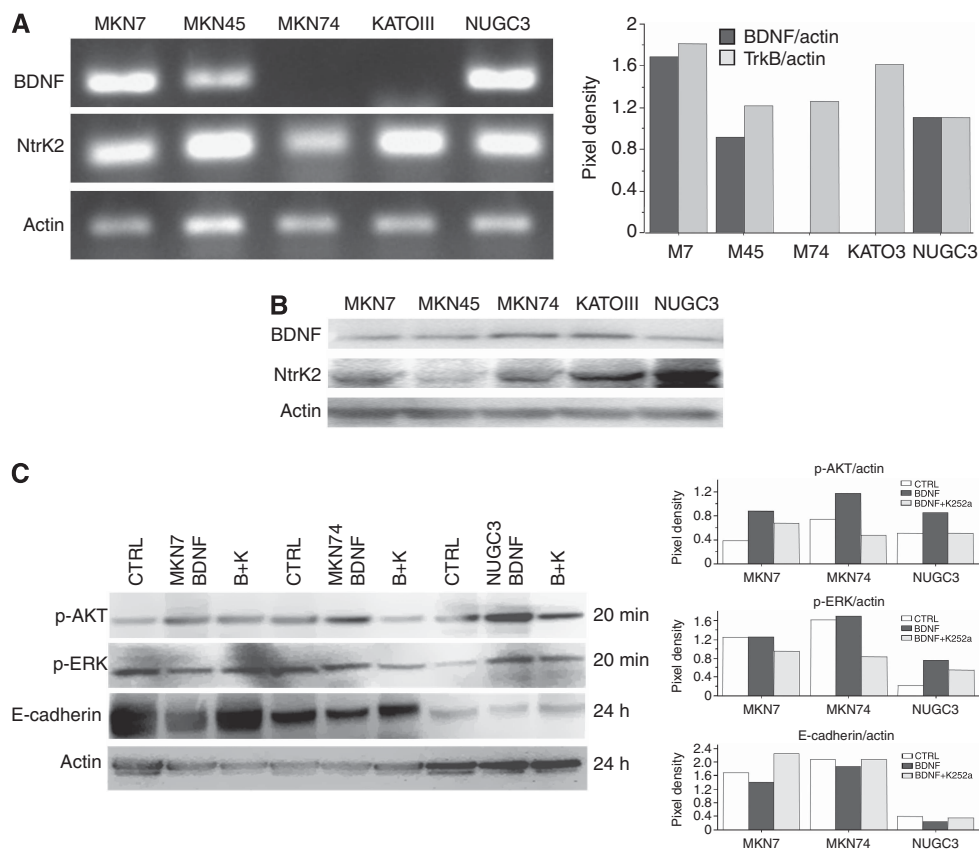


Figure 2. (A) Semi-quantitative RT-PCR analysis to detect BDNF and TrkB transcript expression in gastric cancer cell lines. The panel on the right shows densitometry analysis results. (B) Western blotting analysis to detect BDNF and TrkB protein expression in gastric cancer cell lines. (C) Treatment with BDNF (100 ng ml^{-1}) or K252a (50 nM) followed by BDNF (100 ng ml^{-1}) stimulated p-AKT, p-ERK, and E-cadherin expression in MKN7, MKN74, and NUGC3 cells. The panel on the right shows densitometry analyses of expression levels normalised to actin.

Brain-derived neurotrophic factor promotes migration and invasion in gastric cancer cells expressing TrkB *in vitro*. In wound-healing assays, an increased migratory effect was observed in rhBDNF-treated cells compared with untreated cells, and those treated with K252a followed by rhBDNF inhibited the migratory effect in all three cell lines tested (Figure 3B).

In invasion assays, the capacity of rhBDNF-treated MKN7 and NUGC3 cells to invade through a Matrigel-coated membrane was significantly greater than that of untreated cells (Figure 3C). K252a treatment followed by rhBDNF significantly reduced the number of invading cells in these cell lines. Treatment with K252a without rhBDNF induced a significant reduction in invasiveness compared with that of untreated cells in a part of gastric cancer cell lines. MKN74 cells did not exhibit invasion following rhBDNF treatment (data not shown).

Brain-derived neurotrophic factor induced anoikis resistance in gastric cancer cells expressing TrkB *in vitro*. Because anoikis is known to induce apoptosis by loss of cell adhesion, we evaluated by MTT assay the number of viable MKN7, MKN74, and NUGC3 cells that were floating in low-attachment plates. The BDNF treatment caused an increase in the number of floating-viable gastric cancer cells, which was significantly higher than that of the control cells in all three gastric cancer cell lines tested. In gastric cancer cells treated with K252a followed by rhBDNF, the effect of rhBDNF was inhibited (Figure 3D).

***In vivo* assay.** To assess whether K252a inhibited tumour growth or peritoneal dissemination, we administered NUGC3 cells, a diffuse-type cell line that produces BDNF, intraperitoneally (2×10^6 cells per 1 ml) into nude mice. Peritoneal dissemination

of NUGC3 cells was confirmed by laparotomy 14 days after intraperitoneal administration. Mice with peritoneal dissemination were randomised into two groups: intraperitoneal administration of 1 ml dissolved K252a ($500 \mu\text{g kg}^{-1}$) ($n = 5$) or intraperitoneal administration of 1 ml PBS ($n = 5$) every 3 days. The abdominal wall was closed after administration of K252a or PBS. Finally, we evaluated the tumour size and number of peritoneal dissemination sites, and the mice were killed after 35 days (Figure 4A). The tumour number and size in the K252a-treatment group were significantly lower than that of the control group (Figure 4B-E).

DISCUSSION

The major finding of the present study is that immunohistochemical analysis showed BDNF expression to be significantly increased in gastric cancer tissue compared with adjacent normal mucosa. In cancerous gastric tissue, BDNF was overexpressed at the invasive front of primary gastric cancer compared with the corresponding tumour core. *In vitro* experiments revealed that gastric cancer cell lines expressed BDNF. Although BDNF was initially identified as a protein secreted by neurons that affects nervous system development, recent studies suggest that BDNF is also produced in several types of cancer, including tumours arising from non-neural tissues (Yang *et al*, 2005; Brunetto de Farias *et al*, 2010). The present study is the first to demonstrate BDNF expression in gastric cancer samples and cell lines.

Another major finding of this study is that overexpression of BDNF at the invasive front has significant correlation with vessel

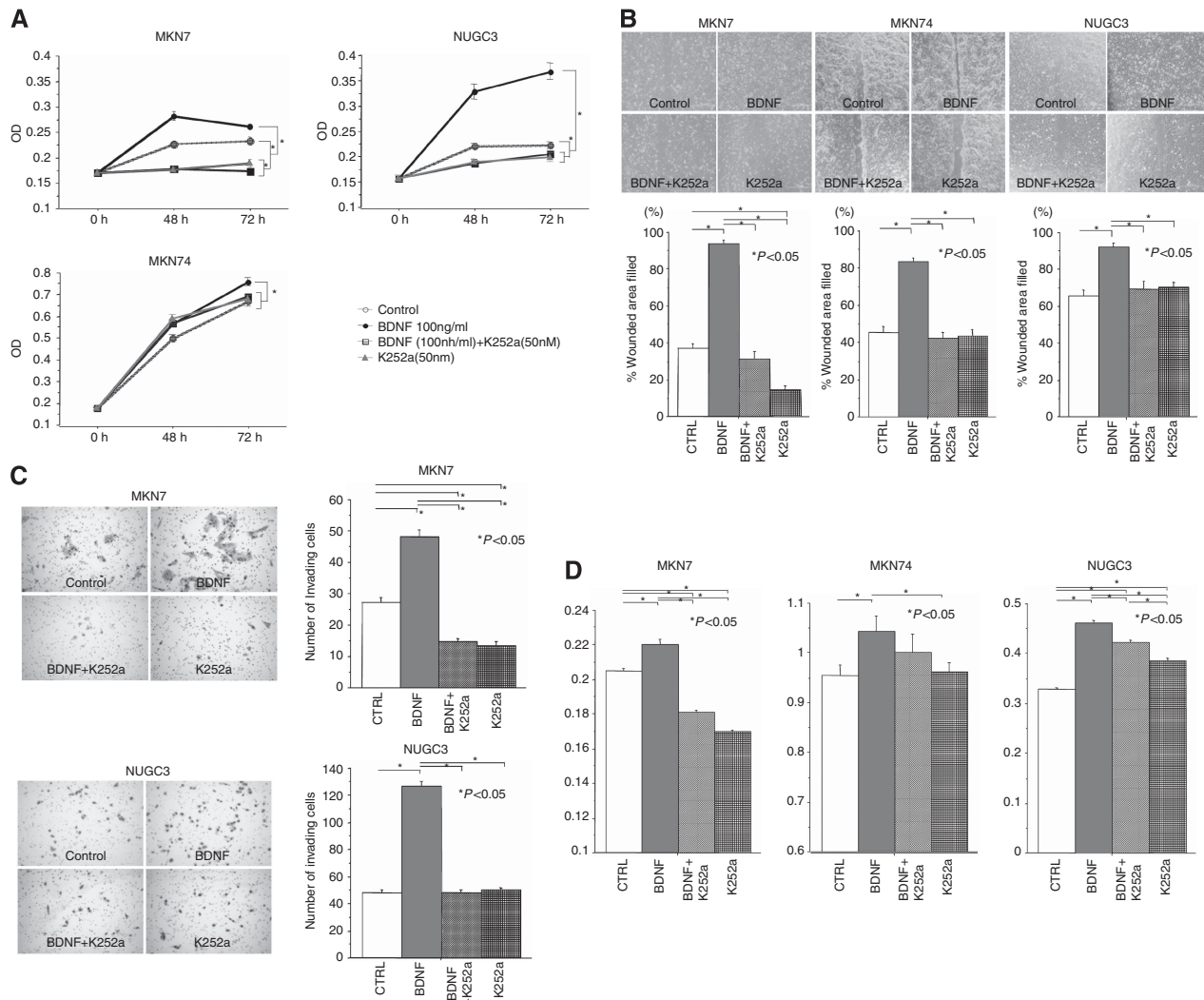


Figure 3. (A) A proliferation assay after 48 and 72 h to investigate the proliferative effects of BDNF (100 ng ml^{-1}), K252a (50 nM), or K252a (50 nM) followed by BDNF treatment (100 ng ml^{-1}) in MKN7, MKN74, and NUGC3 cells. (B) Migration scratch assay to investigate the migratory potential of MKN7, MKN74, and NUGC3 cells after treatment with BDNF (100 ng ml^{-1}), K252a (50 nM), or K252a (50 nM) followed by BDNF treatment (100 ng ml^{-1}). (C) Invasion assays demonstrated that the invasive capability of MKN7 and NUGC3 cells was significantly increased by BDNF treatment (100 ng ml^{-1}) compared with untreated cells, cells treated with K252a (50 nM), or K252a (50 nM) followed by BDNF (100 ng ml^{-1}) (D) Anoikis assay to investigate the anoikis resistance of MKN7, MKN74, and NUGC3 cells after treatment with BDNF (100 ng ml^{-1}), K252a (50 nM), or K252a (50 nM) followed by BDNF treatment (100 ng ml^{-1}). After anoikis induction for 18 h, an anoikis assay was performed and the number of viable floating cancer cells in low-attachment plates was calculated by MTT assay.

involvement, lymph node metastasis, peritoneal dissemination, and poor prognosis in gastric cancer patients. Interestingly, BDNF upregulation at the invasive front compared with the core is similarly significantly associated with poor prognosis. In addition, our study demonstrated that elevated co-expression of BDNF and TrkB at the tumour-invasive front was significantly correlated with lymph node metastasis and poor prognosis in these patients. Several reports described the correlation between the BDNF/TrkB pathway and poor prognosis in other types of cancer (Nakagawara *et al*, 1994; Asgharzadeh *et al*, 2006; Patani *et al*, 2011). Recently, Patani *et al* (2011) demonstrated that higher levels of BDNF transcripts were associated with unfavourable pathological parameters and poor prognosis in breast cancer patients. Our results suggested that BDNF/TrkB expression at the invasive front of primary tumours could be useful as a potential prognostic biomarker for gastric cancer patients and that the BDNF/TrkB pathway might be strongly involved in gastric cancer disease progression.

For several types of cancer, tumour cells at the invasive front are considered to have a more aggressive behaviour compared with those in the more central region (Cianchi *et al*, 2010; Alpizar-Alpizar *et al*, 2011; Tsutsumi *et al*, 2011) and are characterised by a dynamic process referred to as epithelial mesenchymal transition (EMT) (Brabletz *et al*, 2001; Spaderna *et al*, 2006). Epithelial mesenchymal transition is considered to be a transient and reversible process, and represents only one of the several steps required for tumour progression *via* invasion and metastatic spread (Sleeman, 2000), because it has also been implicated in the fundamental steps of tumorigenesis, such as invasion and metastasis (Allan *et al*, 2008). In particular, several studies indicated that TrkB signalling enhanced cancer cell migration and invasiveness *via* EMT-like changes, such as E-cadherin downregulation and vimentin upregulation (Smit *et al*, 2009; Kupferman *et al*, 2010; Dudas *et al*, 2011; Smit and Peeper, 2011; Fujikawa *et al*, 2012). Fujikawa *et al* (2012) demonstrated that E-cadherin expression in TrkB siRNA-transfected cells was higher

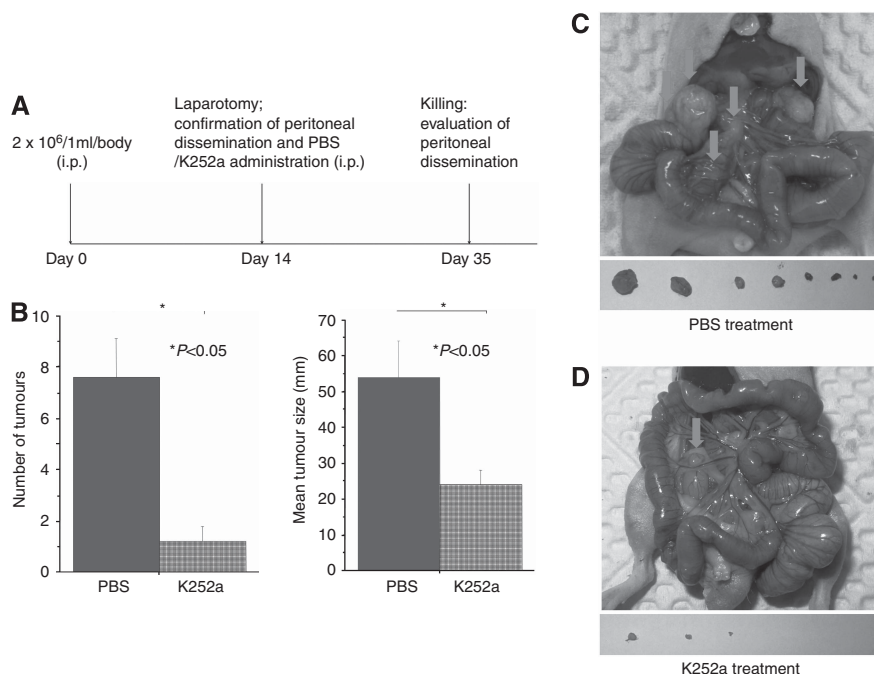


Figure 4. Suppression of peritoneal dissemination in mice by intraperitoneal administration of K252a. (A) Time schedule for establishing peritoneal dissemination, treatment, and evaluation. (B, C) Effect of K252a on peritoneal dissemination was assessed by counting the number of nodules and evaluating tumour size in the mesentery and peritoneal wall compared with controls. Five mice were used in each group. Each value represents mean \pm s.e. (D, E) K252a (5 μ m per 1 ml) suppressed growth of NUGC3-induced nodules in the peritoneum compared with PBS (1 ml) on day 21 after treatment.

than that in control cells and promoted migration and invasiveness, suggesting that TrkB could induce EMT and have an important role in CRC progression. Kupferman *et al* (2010) also demonstrated that enforced TrkB overexpression results in altered expression of molecular mediators of EMT, including E-cadherin downregulation and upregulation of Twist, and was associated with invasiveness in head and neck squamous cell carcinoma. Our study also demonstrated that BDNF treatment downregulated E-cadherin expression in gastric cancer cells. In addition, the BDNF/TrkB axis promoted cell migration and invasiveness in *in vitro* experiments. These results suggest that the BDNF/TrkB pathway might have a pivotal role in the migration and invasiveness of gastric cancer *via* EMT.

In cancer biology, metastasis is a pivotal event that determines the prognosis of patients with malignant disease. Metastasis from the primary site is established through multiple steps, with the first step being detachment of cancer cells from the primary tumour site (Engers and Gabbert, 2000; Fidler, 2002).

Anoikis is an apoptotic cell death that results from loss of or inappropriate cell–matrix interactions (Frisch and Francis, 1994). Dysregulation of anoikis, such as anoikis resistance, is a critical mechanism in tumour metastasis (Eccles and Welch, 2007). During metastasis, epithelial cancers spread to distinct organs through the blood stream. Tumour cells that acquire anoikis resistance can survive after detachment from their primary site and while travelling through the vascular system until they colonise distal organs (Simpson *et al*, 2008; Uekita *et al*, 2008; Kim *et al*, 2012; Taddei *et al*, 2012). In addition, anoikis resistance is important for peritoneal dissemination of gastric cancer and ovarian cancer cells (Uekita *et al*, 2008; Yokoe *et al*, 2010; Masoumi Moghaddam *et al*, 2012). Acquisition of resistance to anoikis is therefore a prerequisite for gastric cancer cells to survive in a detached state before they form metastatic foci and have a critical role in the progression of distant metastasis.

When gastric cancer cells were activated by BDNF in our study, TrkB in turn activated downstream signalling molecules, such as protein kinase B (Akt) and ERK, which are reported to promote cell proliferation and inhibit apoptosis through a number of downstream targets (Li *et al*, 2004; Simpson *et al*, 2007; Kawamura *et al*, 2010). Indeed, our present study revealed that BDNF treatment increased the proliferative activity and conferred anoikis resistance to all three TrkB-expressing gastric cancer cell lines tested. In addition, immunohistochemical analysis demonstrated that disseminated gastric cancer cells of the lymph node and peritoneum expressed both BDNF and TrkB protein in the same pattern as that observed at the primary lesion in the same patient. Interestingly, our *in vivo* study demonstrated that the Trk antagonist K252a suppressed the peritoneal dissemination of BDNF autocrine gastric cancer cells, although limited by the factor that pan-Trk antagonist K252a, which also blocks other Trk (TrkA and TrkC) signalling, might be involved in the *in vivo* effect of K252a. Recently, activation of the BDNF/TrkB pathway was demonstrated to participate in the cellular mechanism for anoikis resistance in several cancer models, such as liver (Zhang *et al*, 2008, 2009), pancreatic (Sclabas *et al*, 2005), and ovarian cancers (Yu *et al*, 2008). Ng *et al* (2012) demonstrated that BDNF/TrkB signalling was deeply involved in anoikis resistance and that K252a harboured potent anoikis-sensitisation activity against nasopharyngeal cancer cells. Our findings here suggest that cancer cells at the invasive front of primary tumours acquired proliferation ability and anoikis resistance *via* the BDNF/TrkB pathway. This mechanism might sustain the anti-apoptotic state of detached cells as they disseminate from the primary tumour to metastatic sites and promote viable lymph node or peritoneal metastasis.

One of the possible roles of BDNF/TrkB in cancer is protecting tumour cells from anticancer therapy. For example, BDNF has been shown to protect neuroblastoma cells against etoposide (Li *et al*, 2011). In CRC, BDNF/TrkB signalling may offer

protection from the antitumour effects of agents targeted to specific growth factor receptors, including anti-EGFR antibodies (Brunetto de Farias *et al*, 2010; de Farias *et al*, 2012). Although our study also evaluated whether K252a treatment induced 5-fluorouracil sensitivity *in vitro*, K252a treatment did not affect the anti-tumour activity of 5-fluorouracil (data not shown). However, because the effects of other anticancer or molecularly targeted drugs on K252a treatment could not be evaluated, there remains the possibility that BDNF/TrkB signalling may have a protective effect against anti-tumoural drugs and have a stimulatory role in cancer cell survival and cancer progression in gastric cancer.

In conclusion, we demonstrate the clinical and biological function of the BDNF/TrkB pathway in gastric cancer and provide the first evidence that the BDNF/TrkB pathway has an important role in invasion and metastasis of gastric cancer. Assessment of the BDNF and TrkB expression status in primary tumours provides clinically useful prognostic information for gastric cancer patients and indicates that the BDNF/TrkB pathway might be a novel therapeutic target for developing gastric cancer treatments.

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