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https://doi.org/10.15017/26360

出版情報：九州大学, 2012, 博士（医学）, 課程博士
バージョン：accepted
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TrkB/BDNF signaling pathway is a potential therapeutic target for pulmonary large cell neuroendocrine carcinoma

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Running Title: BDNF/TrkB are potential therapeutic target for pulmonary LCNEC

Key words: LCNEC; TrkB; BDNF; invasion; tumorigenicity; lung cancer
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Word count: 2995

Figures (5) Table (1)
Abstract

Tropomyosin-related kinase B (TrkB) plays an important role in tumor progression in various kinds of cancers; however, little is known about biological significance of TrkB in human lung cancer, especially large cell neuroendocrine carcinoma (LCNEC). We hereby investigated the expressions of TrkB and its ligand brain-derived neurotrophic factor (BDNF) in clinical specimens and their influences on phenotypes of invasiveness and tumorigenicity for LCNEC. The expressions of TrkB and BDNF analyzed by immunohistochemistry for patients samples with lung cancer (n=104) were significantly higher in neuroendocrine tumor (NET) compared with non-NET. In particular, LCNEC, a subtype of NET, exhibited significantly higher TrkB and BDNF expressions than another NET type: small cell lung cancer (SCLC), and a significant correlation between TrkB and BDNF expressions was noted in LCNEC but not in SCLC. In vitro assay, exogenous BDNF addition enhanced the invasion into matrigels of LCNEC cells, whereas inhibition of TrkB or BDNF suppressed matrix metalloproteinase-2 and -9 activities and the invasiveness. Exogenous BDNF also increased anchor-independent colony formation on soft agar gels for LCNEC, while inhibition of TrkB or BDNF suppressed the anchorage-independency. In vivo experiments, implanted LCNEC cells pretreated with TrkB-siRNA developed no
subcutaneous tumor in all six nude mice, although those with control-siRNA formed
tumors in four of six nude mice. In conclusion, BDNF/TrkB signal is involved in
malignant progression of invasiveness and tumorigenicity for LCNEC, and may be a
potential target for LCNEC without standard therapy.
1. **Introduction**

Lung cancer is the leading cause of death among the malignant tumors worldwide, and most of these deaths are caused by the metastasis of tumor (1). Among several types of lung cancers, neuroendocrine carcinomas: small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC) exhibit especially aggressive phenotypes due to their frequent recurrence and high metastatic potential (2). Based on large-scale clinical trials, the guideline of standard therapies for SCLC has been established, despite the outcome has met with limited success (3). On the other hands, much less is known about LCNEC, and no consensus on therapy has been reached. Therefore, effective target for LCNEC regulation are earnestly desired to be identified.

Tropomyosin-related kinase B (TrkB) is a member of Trk family, functions as a receptor tyrosine kinase for brain-derived neurotrophic factor (BDNF), a member of nerve growth factor family. BDNF and TrkB are necessary for the normal development of the nervous system (4, 5) and lung (6). BDNF and TrkB are up-regulated in a variety of malignant solid tumors, including neuroblastoma (7), bladder (8), pancreatic (9), and ovarian cancers (10). TrkB is also known as a negative prognostic factor for patients with neuroblastoma, Wilms’ tumor, pancreatic, hepatocellular, and gastric
cancers(11-13). When stimulated by BDNF ligand, TrkB induces the activation of downstream AKT (14, 15), ERK (16, 17), HIF-1α (18) signals, which elicits the differential regulation of various cellular activities, such as cell proliferation (19), differentiation (20), apoptosis (21) and invasion (22). Accordingly, TrkB signal contributes to resistance to anoikis, formation of large tumor aggregates and infiltration into lymphatic and blood vessels, resulting in high potential of metastasis (23).

With respect to TrkB in lung cancer, higher expression of TrkB has been shown in non small cell lung cancer and closely correlated with lymph node metastasis (24). It has also reported higher expression of TrkB correlate with overall survival in squamous cell carcinoma (25). Nevertheless, it has not been examined whether TrkB/BDNF pathway is up-regulated and involved in malignant progression for neuroendocrine tumor (SCLC and LCNEC) of lung cancer.

In this study, we investigated the expression and biological significance of TrkB/BDNF signaling for neuroendocrine tumor of lung cancer.

**Materials and Methods**

2.1. **Patients.**

Ninety eight patients with lung cancer received curative surgical resection at
Hamanomachi Hospital from August 1995 to August 2009. Six patients with lung
cancer underwent curative surgery at Kyushu Kosei Nenkin Hospital from September
2004 to March 2009. All patients have received no chemotherapy or radiation therapy
prior to surgery. All patients with LCNEC and SCLC were selected because of the small
number of cases while patients with other histological type were quite randomly chosen.
The clinicopathological profiles of LCNEC and other histological types are shown in
Table 1. Although significant difference is observed only in venous invasion, there were
no significant difference in many other clinicopathological factors such as age, gender,
tumor size, lymphnode metastasis, lymphnode invasion, and TNM stage among
subgroups. The hospital’s ethics committee approved the study protocol. All patients
were given full explanations and gave written informed consent before treatment.

2.2. Cell lines

We used LCNEC cell lines (NCI-H460, NCI-H810), lung adenocarcinoma cell
line (A549), SCLC cell line (SBC-3) and lung large cell carcinoma cell line (LU99).
NCI-H460, A549, SBC-3 and LU99 were cultured in RPMI 1640 supplemented with
10% fetal bovine serum (FBS). NCI-H810 was cultured in DMEM:F12 (American Type
Culture Collection) supplemented with 5% FBS, 0.005 mg/ml insulin, 0.01 mg/ml
transferrin, 30 nM sodium selenite, 10 nM beta estradiol, 10 nM hydrocortisone, 2 mM L-glutamine.

2.3 Small interfering RNA transfection

Small interfering RNA targeting TrkB (Dharmacon, Lafayette, CO, USA), BDNF, matrix metalloproteinase (MMP)-2, or MMP-9 were transfected into NCI-H460 and NCI-H810 cells (final concentration: 50 nM) by using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Non-targeting siRNA (Dharmacon) was used as the negative control.

2.4. Real-time RT-PCR

Total RNA was isolated with the High Pure Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany), and reverse-transcribed using the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Reactions were performed with SYBR Premix EX Taq II (Takara Bio, Shiga, Japan) on a DNA Engine Opticon 2 system (MJ research, Waltham, MA, USA). The amount of each target gene was normalized to the level of β-actin. The primers used were as follows: MMP-2, forward, 5’-TGATCTTGACCAGAATACCATCGA-3’ and reverse,
5′-GGCTTGCAGGGAAGGAAGTT-3’; MMP-9, forward,
5′-TGGGCCTACGTGACCTATGACAT-3’ and reverse,
5′-GCCAGGGGACCTCCACTCCTC-3’; ACTB, forward,
5′-TTGTTACAGGAAGTCCCTGCG-3’ and reverse,
5′-ATGCTATCACCTCCCCTGTGTG-3’

2.5. Immunohistochemistry

Paraffin sections of lung cancer were deparaffinized and rehydrated routinely. The sections were immersed with 3% H$_2$O$_2$ and 10% goat serum and incubated with a primary antibody for TrkB (sc-8316, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:100) or BDNF (sc-546, Santa Cruz, 1:100) at 4°C overnight. The samples were incubated with HISTOFINE simple stain MAX-PO (R) (Nichirei, Tokyo, Japan) and finally visualized by 3,3’-diaminobenzidine (DAB) with hematoxylin-counterstain. Both the intensity and percentage of TrkB and BDNF expression were assessed as follows: intensity score 0: absence of staining, 1: weakly stained, 2: moderately stained, 3: strongly stained; percentage score 0: absence of positive cells, 1: 0.1-33% of cells positive, 2: 33.1-66% of cells positive, 3: >66% of cells positive; total score (ranging from 0 to 9) was calculated by multiplying intensity score and percentage score.
2.6. Cell immunofluorescence

NCI-H460 and NCI-H810 cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The samples were incubated with a primary rabbit polyclonal antibody for TrkB (sc-8316, 1:100) or BDNF (sc-546, 1:100) at 4°C overnight, following by a secondary Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen, 1:1000). The nuclei were counterstained with 4-6-diamidino-2-phenylindole (DAPI) (Invitrogen), and protein expression level of TrkB and BDNF was evaluated by fluorescence intensity under fluorescence microscopy (Carl Zeiss, Tokyo, Japan), as previously, described (26).

2.7. Western blot analysis

Cultured cells were lysed in SDS-Buffer, proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies for TrkB (sc-8316, 1:200), BDNF (sc-546, 1:200), MMP-2 (sc-10736, Santa Cruz, 1:200), MMP-9 (sc-6840, Santa Cruz, 1:200), or α-tubulin (Sigma-Aldrich, St. Louis, MO, USA, 1:1000) at 4°C overnight, followed by
peroxidase-labeled secondary antibodies at 37°C. Immunoblots were identified using
the ECL Prime Western Blotting Detection System (GE Healthcare Life Sciences,
Buckinghamshire, UK) with a Molecular Imager FX (Bio-Rad Laboratories, Hercules,
USA).

2.8. Cell invasion assay

The invasiveness of LCNEC cells was assessed by invasion assay described by
previously (27). In brief, cells (2×10^5) were added to the upper chamber and incubated
for 24 hours. Total number of cells migrated to the lower side of the filter was counted
under a light microscope.

2.9. In vivo Xenograft tumor model

Five-week-old female athymic nude mice (BALB/c nu/nu) were purchased
from Charles River Laboratories Japan (Kanagawa, Japan) and acclimated for two
weeks. All animal procedures were approved by the Animal Care and Use Committee at
Kyushu University. NCI-H810 cells transfected with TrkB-targeting siRNA or
non-targeting control siRNA were subcutaneously implanted into flank regions (5×10^6
cells in PBS per mouse) of the nude mice (n=6 in each). The tumor size was determined
twice a week and the tumor volume was calculated by the following formula: length ×
(width)$^2 \times 0.5$ (cm$^3$).

2.10. Statistical analysis

All data were represented as mean ± standard deviations (SD). $\chi^2$ test was
applied to analyze the relationship between clinicopathological parameters. Student’s
t-test was used for the comparison of mean values between two groups. Survival curves
were plotted by Kaplan-Meier method and analyzed using two-sided log-rank tests. A
value of $P< 0.05$ was considered significant.

2. Results

3.1. Clinicopathological characteristics of patients with lung cancer

Total 104 cases of lung cancer were histologically diagnosed as follows.
Neuroendocrine tumor (NET) included LCNEC (n=11) and SCLC (n=8), while
non-neuroendocrine tumor (non-NET) consisted of large cell carcinoma (n=10),
adenocarcinoma (n=20) and squamous cell carcinoma (n=55). Overall survival in NET
and non-NET are shown in Fig. 1A. The 5-year survival rates for patients with NET and
non-NET were 43.5% and 77.3%, respectively and overall survival in NET was
significantly lower than that in non-NET.
3.2. *TrkB* and *BDNF* expression in lung cancer tissues of patients

We investigated TrkB and BDNF expression in lung cancer tissues of patients by immunohistochemistry. The expression of TrkB was detected in 91 (88%) out of 104 lung tumors: higher expression (scores of 4, 6, or 9) in 38 cases (37%) and lower expression (scores of 0, 1, 2, or 3) in 66 cases (64%). The expression of BDNF was detected in 88 (85%) out of 104 lung tumors: higher expression (scores of 4, 6, or 9) in 41 cases (39%) and lower expression (scores of 0, 1, 2, or 3) in 63 cases.

We evaluated TrkB and BDNF expression among NET, non-NET and normal bronchial epithelial (NBE) tissues distant from tumor area by immunohistochemistry. The % scores of TrkB and BDNF were significantly higher in NET or non-NET compared with NBE (Fig. 1B). The intensity scores of TrkB and BDNF were significantly higher in NET than NBE or non-NET and there was no difference between non-NET and NBE (Fig. 1C). Accordingly, the total scores of TrkB and BDNF were significantly higher in NET compared with either non-NET or NBE (Fig. 1D). Samples of TrkB and BDNF expression (total score of 0, 3, 6 and 9) in lung cancer are shown in Fig. 1E and 1F, respectively. A high correlation was noted for co-expression of BDNF and TrkB in NET (*P*<0.001) (Fig. 1G).
We further analyzed the expression status of TrkB and BDNF separately for SCLC and LCNEC subtypes of NET lung cancers. Total scores of TrkB and BDNF were significantly higher in LCNEC than SCLC (Fig. 2A). The correlation of TrkB and BDNF in SCLC and LCNEC, respectively was also studied. A significant correlation between BDNF and TrkB expression was noted for LCNEC (P=0.023), but not for SCLC (P=0.17) (Fig. 2B). These data suggested BDNF and TrkB played a more important role in LCNEC than SCLC. Therefore, we used LCNEC cell lines to understand the biologic consequences of BDNF/TrkB signaling in vitro.

3.3. BDNF/TrkB signaling enhances invasive phenotype of LCNEC cells

We hypothesized BDNF/TrkB signaling may contribute to some malignant phenotype for LCNEC and examined the role of BDNF/TrkB on invasiveness of LCNEC. TrkB and BDNF expression in lung cancer cell lines was analayzed by Western blotting (Fig. 3A). LCNEC cell lines (NCI-H460 and NCI-H810 showed high TrkB and BDNF expression. TrkB and BDNF expression in NCI-H460 and NCI-H810 was also confirmed immunocytochemistry (Fig. 3B). Addition of recombinant human BDNF (Peprotech, Rocky Hill, NJ, USA) or TrkB tyrosine kinase inhibitor K252a
(Alomone Labs, Jerusalem, Israel) to culture medium did not affect the proliferation of these cells (Fig. 3C). In contrast, exogenous BDNF treatment (10, 100 and 1000 ng/ml) significantly enhanced matrigel invasion of these cells (Fig. 3D) in a dose-dependent manner. Reversely, K252a (500, 1000 nM) significantly decreased the invasion in NCI-H460 and NCI-H810 cells (Fig. 3E). As shown in Fig. 3F, enhanced cell invasion by BDNF treatment was interrupted by K252a, suggesting the invasive potential of LCNEC cells may be mediated through BDNF/TrkB signaling. To verify the direct link of BDNF/TrkB signaling to the phenotypic alterations in LCNEC, we carried out the inhibition experiments using TrkB-siRNA or BDNF-siRNA LCNEC cell lines. Western blotting shows the inhibition of TrkB and endogenous BDNF by the siRNA knockdown (Fig. 3G). TrkB and BDNF-siRNAs significantly inhibited the invasion of NCI-H460 and NCI-H810 cells (Fig. 3H). Exogenous BDNF addition had little effect on the invasion of LCNEC cell lines transfected with TrkB-siRNA, but reversed the invasive ability of LCNEC cells downregulated by BDNF-siRNA transfection. TrkB and BDNF co-knockdown significantly inhibited the invasion even in the presence of exogenous BDNF.

3.4. BDNF/TrkB-induced invasion is mediated through MMP-2 and MMP-9
To explore the underlying mechanism mediating invasion by BDNF/TrkB signaling in LCNEC cells, we examined the effect of BDNF ligand on the activities of MMP-2 and MMP-9 in NCI-H460 and NCI-H810 cells. The increased expression and activities of MMP-2 and MMP-9 by BDNF treatment were identified in NCI-H460 and NCI-H810 by quantitative RT-PCR (Fig. 4A), Western blotting (Fig. 4B) and zymography (Fig. 4C). On the other hand, TrkB or BDNF knockdown significantly decreased the expression of MMP-2 and MMP-9 (Supplementary Fig. S1). The decreased expression in MMP-2 and MMP-9 with TrkB-siRNA transfection was not recovered by exogenous BDNF treatment (Fig. 4D and 4E), whereas the decreases with BDNF-siRNA were compensated by exogenous BDNF in LCNEC cells. We further investigated the effect of MMP-2 or MMP-9 knockdown on the invasion of LCNEC cells. In a preliminary experiment (Supplementary Fig. S2), MMP-2-siRNA or MMP-9-siRNA transfection induced over 80% inhibition of MMP-2 or MMP-9, respectively. MMP-2 or MMP-9 knockdown significantly inhibited the invasion of NCI-H460 and NCI-H810 (Fig. 4F) irrespective of exogenous BDNF addition. MMP-2 and MMP-9 concurrent knockdown inhibited the invasion more than MMP-2 or MMP-9 knockdown alone. These data suggest enhanced cell invasion by BDNF/TrkB signaling is through the upregulation of MMP-2 and MMP-9 pathway in LCNEC.
3.5. BDNF/TrkB signaling augments colony formation in LCNEC cells

We next investigated the role of BDNF/TrkB signaling on anchorage-independent growth of LCNEC cells. As shown in Fig. 5A and 5B, TrkB and BDNF-siRNAs significantly inhibited the colony formation of NCI-H460 and NCI-H810 cells. Exogenous BDNF addition had little effect on the colony formation of LCNEC cell lines transfected with TrkB-siRNA, but reversed the decrease in colony formation of LCNEC cells transfected with BDNF-siRNA. TrkB and BDNF co-knockdown significantly inhibited the colony formation even in the presence of exogenous BDNF. These results suggest BDNF/TrkB signaling mediate the anchorage-independent growth of LCNEC cells.

3.6. Inhibition of TrkB suppresses tumorigenicity in vivo

To determine whether the observed anchorage-independent growth by BDNF/TrkB signaling could be reflected in vivo, we investigated tumorigenicity of LCNEC cells with or without TrkB silencing in athymic nude mice. Subcutaneous tumors were developed in four out of six mice after implantation of cells transfected with control siRNA (Fig. 5C and 5D). In contrast, all the mice received cells transfected
with TrkB-siRNA showed no sign of tumor development throughout the observation period until three months after the cell implantation. There were significant differences in tumor growth between TrkB-siRNA and control siRNA groups (Fig. 5D). We confirmed TrkB and BDNF were highly expressed in tumor cells transfected with control siRNA (Fig. 5E).

3. Discussion

The present study was firstly designed to investigate biological significance of BDNF/TrkB signaling for NET: LCNEC and SCLC of lung cancer. We at first examined BDNF and TrkB expression status in lung cancer specimens. Both TrkB and BDNF expression levels in LCNEC were significantly higher than SCLC or non-NET tumor types of lung cancer. Furthermore, a significant correlation between BDNF and TrkB expressions was noted in LCNEC, but not in SCLC. It is sometimes difficult to histologically differentiate between LCNEC and SCLC, because they share several histological features, such as rosette formation, moulding of nuclei, and lack of apparent glandular formation and keratinization (28). Then, BDNF/TrkB signaling may be a diagnostic aid for differentiation between LCNEC and SCLC. However, whether low TrkB and BDNF expression in LCNEC correlates with good prognosis was not
observed. The reason may be that there were high TrkB and BDNF expression in all 11 patients with LCNEC. The incidence of LCNEC is only about 2% of resected lung cancers (29, 30), and the number of LCNEC patients in this study could not be over 11 cases. To define whether low TrkB and BDNF expression correlate with good prognosis in LCNEC, would require further investigation in a larger cohort of patients.

Metastasis is the most critical determinant for postoperative prognosis of patients with lung cancer. We investigated the significance of BDNF/TrkB signaling in LCNEC progression, firstly with respect to the effect on invasion. Degradation of the extracellular matrix and the basement membranes by proteases promotes the detachment of tumor cells, their crossing of tissue boundaries, and invasion into adjacent blood or lymphatic vessels and tissue compartments. Recent studies have demonstrated the important role of MMPs in cancer invasion and metastasis for a variety of solid malignant tumors (31). Especially, MMP-2 and MMP-9 (72 kDa and 92 kDa type IV collagenases or gelatinase A and gelatinase B) are associated with the malignant phenotype of tumors because of their unique ability to degrade type IV collagen (32). In the present study, BDNF/TrkB signaling enhanced invasive phenotype of LCNEC cells, which is consistent with the previous reports in non-SCLC (24) and head and neck tumor (22). We hypothesized MMP-2 and MMP-9 may be downstream mediators for
BDNF/TrkB signals in LCNEC cells, and verified this hypothesis with the data that exogenous BDNF increased MMP-2/-9 activities and cell invasion and that TrkB-siRNA and MMPs-siRNA abolished the enhanced MMP activities and invasion by BDNF.

Once cancer cells invade into blood or lymphatic vessels, the attachment to metastatic niche and anchor-independent growth are required to form the metastatic tumors. Thus, we investigated the effect of BDNF/TrkB signaling on anchor-independent colony formation of LCNEC cells, and found the anchorage-independent growth was enhanced by exogenous BDNF and suppressed by TrkB inhibition, despite the anchor-dependent (dish-attached) growth of LCNEC cells was not affected by the BDNF/TrkB modulation (Fig. 3C). To determine whether the observed anchorage-independent growth by BDNF/TrkB signaling could be reflected in vivo, we implanted TrkB-silenced LCNEC cells in athymic nude mice, and observed inhibition of TrkB completely disturbed the tumor formation of the LCNEC cells in vivo. The prophylactic effect of TrkB-silencing in this mouse model suggests BDNF/TrkB pathway may be a potential therapeutic target for LCNEC.

Our data indicate that the TrkB and BDNF pathway provides critical signals for not only MMP-mediated invasion but also for survival and tumor formation of LCNEC,
as shown in Supplementary Fig. S3. In conclusion, our data indicate BDNF and TrkB are overexpressed in LCNEC type of lung cancer, and BDNF/TrkB signaling is involved in invasiveness and tumorigenicity of LCNEC.

Conflict of interest statement

None declared.

Acknowledgments

This study was supported by a General Scientific Research Grant (23592065) from the Ministry of Education, Culture, Sports and Technology of Japan.
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Figure Legends

Fig. 1. TrkB and BDNF expression in lung cancer of patients.

(A) Kaplan-Meier survival curve for neuroendocrine tumor (NET) type lung cancers (n=19, LCNEC and SCLC) and non-neuroendocrine tumor (non-NET) type lung cancers (n = 85, large cell carcinoma, adenocarcinoma and squamous cell carcinoma).

(B), (C), (D) Lung cancer tissues of patients were immunostained with an antibody for human TrkB or BDNF, and the expression level was evaluated as an index of total score (= % score × intensity score) in “Materials and Methods”. TrkB and BDNF expression levels evaluated as an index of percentage scores (B), intensity scores (C), total scores (D) were compared between normal bronchial epithelial (NBE), non-NET and NET. *P<0.001. NS, not significant.

(E) Immunohistochemical staining for TrkB in lung cancer specimens, adenocarcinoma (upper left); squamous cell carcinoma (upper right, lower left); LCNEC (lower right). Original magnification: ×400.

(F) Immunohistochemical staining for BDNF in lung cancer specimens, adenocarcinoma (upper left, lower right); squamous cell carcinoma (upper right); LCNEC (lower left). Original magnification: ×400.
(G) A correlation between TrkB and BDNF expression in NET (n=19).

**Fig. 2.** TrkB and BDNF expression and its correlation with prognosis for small cell-type and LCNEC-type lung cancers.

(A) TrkB and BDNF expression levels evaluated as an index of total scores were compared between SCLC and LCNEC. *P<0.001.

(B) A correlation between TrkB and BDNF expression in SCLC (n=8, left) and LCNEC (n=11, right).

**Fig. 3.** BDNF/TrkB signal enhances invasive phenotype of LCNEC cells.

(A) Protein lysates extracted from lung cancer cells (NCI-H460, NCI-H810, LU99, A549, SBC-3) were subjected to Western blot analysis for TrkB and BDNF. Mouse brain lysate was used as the positive control.

(B) LCNEC cells were immunostained with fluorescence-labeled (green) TrkB or BDNF antibodies, followed by counterstained with DAPI (blue). Histograms represent the expression intensities of TrkB or BDNF (green) and DAPI (blue). Original magnification: ×200.

(C) NCI-H460 and NCI-H810 were seeded onto 96-well plates at a density of 5000
cells/well and incubated with recombinant human BDNF or a TrkB tyrosine kinase inhibitor K252a for 24 and 48 hours. Cell proliferation was determined by the absorbance counts at 492 nm (ref. 620nm) using Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan). BDNF or K252a treatment did not affect the growth of NCI-H460 and NCI-H810 cells analyzed by cell proliferation assay (D), (E), (F) NCI-H460 and NCI-H810 cells were seeded onto matrigel-coated invasion chambers. After incubation for 24 hours in the presence of BDNF (10, 100 or 1000 ng/ml) (D), K252a (100, 500 or 1000 nM) (E), and BDNF (100ng/ml) and K252a (1000nM) (F), migrated cells were stained with Diff-Quick reagent and quantified by bright-field microscopy. Error bars represent standard deviations. *P<0.05. **P<0.001. Original Magnification: ×100.

(G) NCI-460 and NCI-H810 were transfected with the indicated siRNA (50 nM), and 48 later protein lysates (60 μg) were extracted from cells and subjected to Western blot analysis.

(H) NCI-H460 and NCI-H810 cells were transfected with the indicated siRNA and then seeded onto matrigel-coated invasion chambers. After incubation with BDNF (100 ng/ml) for 24 hours, migrated cells were quantified by bright-field microscopy. Error bars represent standard deviations. *P<0.05. **P<0.01. NS, not significant.
Fig. 4. BDNF/TrkB signal-induced invasion is mediated through MMP-2 and MMP-9 in LCNEC cells.

(A), (B), (C) Quantitative RT-PCR (A), Western blotting (B) and gelatin zymography using a gelatin-zymography kit (Primary Cell, Sapporo, Japan) (C) represent the upregulated expression and activities of MMP-2 and MMP-9 by BDNF stimulation in NCI-H460 and NCI-H810 cells.

(D) (E) NCI-H460 (D) and NCI-H810 (E) were incubated with BDNF (100 ng/ml) for 24 hours after transfection with TrkB-siRNA and BDNF-siRNA. Total RNA was extracted from the cells and subjected to quantitative RT-PCR for MMP-2 and MMP-9 expression.

(F) NCI-H460 and NCI-H810 cells were transfected with the indicated siRNA and then seeded onto matrigel-coated invasion chambers. After incubation with with BDNF (100 ng/ml) for 24 hours, migrated cells were quantified by bright-field microscopy. Error bars represent standard deviations. *P<0.05. **P<0.01. §P<0.005. §§P<0.001. NS, not significant.

Fig. 5. BDNF/TrkB signal augments colony formation in vitro and tumorigenecity in
*vivo* for LCNEC cells.

(A), (B) NCI-H460 (A) and NCI-H810 (B) cells were transfected with the indicated siRNA. Cells were resuspended in a 0.35% agar solution, and 0.7% agar layer without cells was overlayed. After incubation with BDNF (100 ng/ml) for 14 days, the colonies were stained by crystal violet and counted under a bright microscopy. Error bars represent standard deviations. *P<0.05. **P<0.01. §P<0.005. §§P<0.001. NS, not significant.

(C), (D) NCI-H810 cells were transfected with control-siRNA or TrkB-siRNA, and then implanted into flank regions of athymic nude mice. Subcutaneous tumor formation was observed in mice with control-siRNA, but not with TrkB-siRNA (C). Tumorigenic ability evaluated by tumor volume (D) was significantly inhibited in TrkB-siRNA group (n=6) compared with the control (n=6). Error bars represent standard deviations.

*P=0.025, **P=0.019, ***P=0.014.

(E) TrkB (left) and BDNF (right) expressions were confirmed by immunohistochemistry in tumor tissues of nude mice implanted with NCI-H810 cells with control-siRNA. Original magnification: ×400.
Table 1. Clinicopathological profiles of LCNEC, SCLC and non-NET in patients with lung cancer.

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Postoperative therapy
- 3   0   35  38  0.055
+ 8   8   50  66

Postoperative recurrence
Without 5   4   60  69  0.15
With   6   4   25  35

LCNEC; large cell neuroendocrine carcinoma, SCLC; small cell lung carcinoma, non-NET; non neuroendocrine tumor (large cell carcinoma, adenocarcinoma and squamous cell carcinoma)

*Significant
**Supplementary Figure Legends**

**Supplementary Fig. S1.** TrkB or BDNF knockdown significantly decreased the expression of MMP-2 and MMP-9.

NCI-H460 and NCI-H810 were transfected with the indicated siRNA (50 nM) and then incubated with BDNF (100 ng/ml) for 24 hours. Cells were collected and quantitative RT-PCR was performed to determine the induction of MMP expression. Error bars represent standard deviations. *P<0.05. **P<0.01.

**Supplementary Fig. S2.** MMP-2 and MMP-9 knockdown by siRNA transfection.

NCI-460 and NCI-H810 were transfected with siRNA targeting MMP-2 and MMP-9, and mRNA of MMP-2 and MMP-9 were analyzed by quantitative RT-PCR.

**Supplementary Fig. S3.** Schematic BDNF/TrkB signaling model for invasiveness and tumorigenicity of LCNEC cells.

BDNF and its receptor TrkB are overexpressed in LCNEC cells. Secreted BDNF binds to TrkB in an autocrine fashion, and activated TrkB signal enhances cell invasion, anchorage-independent growth and tumorigenicity. Exogenous BDNF from stromal cells also interacts and activates TrkB pathway. K252a: inhibits phosphorylation of
TrkB tyrosine kinase.
Supplementary Figure S1

NCI-H460

Fold Change MMP mRNA

- siControl
- siTrkB
- siEDNF

NCI-H810

Fold Change MMP mRNA

- siControl
- siTrkB
- siEDNF

MMP-2
MMP-9
Supplementary Figure S2

Fold Change MMP-2 mRNA

NCI-H460

siControl

siMMP2

81% reduction

NCI-H810

siControl

siMMP2

88% reduction

Fold Change MMP-9 mRNA

NCI-H460

siControl

siMMP9

85% reduction

NCI-H810

siControl

siMMP9

86% reduction