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https://doi.org/10.15017/1500549

Expression of TrkB and BDNF is associated with poor prognosis in non-small cell lung cancer

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Abstract

High expression levels of TrkB and BDNF are associated with aggressive malignant behavior in tumor cells and a poor prognosis in patients with various types of cancer. In this study, we aimed to identify the relationship between TrkB and BDNF expression and clinicopathological variables and prognosis in non-small cell lung cancer (NSCLC). We evaluated TrkB and BDNF expression in the tumor cells of 102 NSCLC patients by immunohistochemistry. Out of all clinicopathological factors examined, only vascular invasion was significantly correlated with TrkB (P=0.010) and BDNF (P=0.015) expression. TrkB-positive tumors had significantly worse disease-free survival (P=0.0094) and overall survival (P=0.0019) than TrkB-negative tumors, and TrkB expression was an independent prognostic factor for disease-free survival (HR 3.735, 95%C.I. 1.560–11.068, P=0.002) and overall survival (HR 4.335, 95%C.I. 1.534–15.963, P=0.004) in multivariate analysis. Finally, our analysis revealed that co-expression of TrkB and BDNF conferred poorer prognosis compared with overexpression of either protein alone. Our results indicate that expression of TrkB and BDNF is associated with poor prognosis in NSCLC patients.

Key words: TrkB; BDNF; survival; prognosis; invasion; lung cancer

Introduction

Lung cancer is a most common cause of cancer-related death worldwide. The majority of lung cancer patients present at advanced stage with a poor prognosis. Even patients with early-stage lung cancer who undergo curative surgical resection often die from recurrent disease and distant metastases. The long-term survival rate for lung cancer patients still remains low [1].

Tropomyosin-related kinase B (TrkB) is a member of the Trk family, and functions as a receptor tyrosine kinase (RTK). TrkB is highly expressed in cells of neural origin, and is involved in the maintenance and development of neurological tissue [2,3]. Brain-derived neurotrophic factor (BDNF) is a ligand of TrkB. Upon BDNF binding, TrkB and its downstream signaling are activated [2,4]. In addition, TrkB is thought to be a key regulator of oncogenesis and tumor progression. Recent preclinical studies have shown that TrkB is highly expressed in anoikis-resistant cells, and TrkB-expressing cells form highly invasive and metastatic tumors [5,6]. TrkB is a regulator of migration and epithelial-to-mesenchymal transition [7], and TrkB activation by BDNF was shown to enhance the proliferation and survival of transitional cell carcinoma cell lines [8].

Overexpression of TrkB has been reported in various malignancies. In neuroblastoma, patients whose tumors express elevated levels of TrkB and BDNF have a poor prognosis [9, 10]. High expression levels of TrkB and BDNF are thought to be associated with more aggressive malignant behavior and a poor prognosis in human cancer, including pancreatic cancer, Wilms' tumor, colon cancer, breast cancer, gastric cancer and hepatocellular cancer [11-17]. Therefore, TrkB might play an important role in the progression of malignant tumors. Although the evidence for TrkB being an important prognostic factor has been accumulated in various types of cancer, the clinical and prognostic significance of TrkB and BDNF expression has not been well evaluated in patients with lung cancer.

In this study, we investigated the expression of TrkB and BDNF immunohistochemically in several histological types of surgically resected lung cancers. We identified the relationship

between TrkB and BDNF expression and clinicopathological variables and prognosis in non-small cell lung cancer (NSCLC).

Materials and Methods

Patients and sample collection

In this retrospective study, we analyzed specimens from 102 patients with NSCLC (57 squamous cell carcinoma, 36 adenocarcinoma, 9 large cell neuroendocrine carcinoma: LCNEC) who had undergone surgical resection for lung cancer at the Department of Surgery and Science, Kyusyu University Hospital, from January 2003 to January 2011. Patients with LCNEC who had undergone surgery from 2004 to 2011, patients with squamous cell carcinoma who had undergone surgery from 2003 to 2008, and patients with adenocarcinoma who had undergone surgery in 2005 were all consecutive cases.

Surgical specimens were fixed in neutral-buffered formaldehyde, and processed for histopathological and immunohistochemical evaluation. The patients' characteristics are summarized in Table 1. Clinicopathological factors, including age, sex, smoking history, extent of differentiation, vascular invasion, lymphatic invasion, and TNM staging were evaluated. The treatment of patients (surgical procedure and adjuvant therapy) was also reviewed. There were 72 males and 30 females, with a mean age of 66.3±10.19 years. Histological subtype of tumors and pathological stage were classified according to the WHO 2004 classification [18] and UICC guidelines of TNM classification, respectively [19]. This study was approved by the Ethics Committee of Kyusyu University.

Immunohistochemistry

The following primary antibodies were used: TrkB (sc-8316, Santa Cruz Biotechnology, Santa Cruz, CA, USA), BDNF (sc-20981, Santa Cruz Biotechnology).

Paraffin sections of surgically resected specimens were routinely deparaffinized and rehydrated. The sections were incubated overnight at 4°C with primary rabbit polyclonal

antibodies against TrkB (1:100 dilution) and BDNF (1:50 dilution), then were incubated with second antibody conjugated with streptavidin-biotin peroxidase (Histofine SAB-PO kit, Nichirei, Tokyo, Japan), and visualized with 3,3'-diaminobenzidine (DAB). Normal brain and kidney sections were used as positive controls for TrkB [7], while normal brain and skin sections were used as positive controls for BDNF [7]. Parallel negative controls omitting primary antibody were also performed, and did not show appreciable background staining. All the immunoreactions were separately evaluated by two investigators (K.O. and T.H) without knowledge of patients' clinical records. TrkB and BDNF staining was weak or non-existent in normal lung and bronchus tissue. The bronchial smooth muscle tissue of normal lung showed weak TrkB and BDNF staining (data not shown). Tumor cells with brown staining in the membranes or cytoplasm were regarded as positive. We classified staining as positive or negative, which we scored as follow: intensity (0=negative, 1=weak, 2=intense), and percentage of positive tumor cells (1=1–50%, 2=51–75%, 3= ≥76%), the scores of each sample were multiplied to give a final score of 0, 1, 2, 3, 4, or 6 [20].

Statistical analysis

Average scores were expressed as mean ± standard error of the mean. Spearman's rank correlation coefficient was used to analyze the correlation between the expression of TrkB and BDNF. Chi-square tests were used to analyze the correlation between clinicopathological factors and TrkB or BDNF immunoreactivity. Disease-free survival times were measured from the date of surgery to the appearance of local or distant tumor progression. Overall survival times were measured from the date of surgery to death or last follow-up. Disease-free survival and overall survival were evaluated using the Kaplan-Meier method. The log-rank test was used to compare the cumulative survival time in each patient group, and the Cox proportional hazard model was used to analyze univariate and multivariate hazards ratios for the study parameters. Variables with significant *P* values in univariate analysis were used for multivariate analysis. *P* values less than 0.05 were considered to be statistically significant. JMP version 9 (SAS Institute, Inc, Cary,

NC, USA) software was used for all analyses.

Results

TrkB and BDNF expression in lung cancer tissue

Representative images of positive and negative expression of TrkB and BDNF are shown in Figure 1. Seventy seven of 102 (75.5%) NSCLC samples were positive for TrkB. Eighty-four of 102 (82.4%) NSCLC samples were positive for BDNF. Positive rate of TrkB expression was 73.7%, 75.0%, and 88.9% in squamous cell carcinoma, adenocarcinoma, and LCNEC, respectively. The average score of TrkB expression was 2.65 ± 0.28, 2.39 ± 0.34 and 3.33 ± 0.62 in squamous cell carcinoma, adenocarcinoma, and LCNEC, respectively, while the positive rate of BDNF expression was 82.5%, 77.8% and 100% in squamous cell carcinoma, adenocarcinoma, and LCNEC, respectively. The average score of BDNF expression was 3.37 ± 0.29, 2.58 ± 0.33 and 3.77 ± 0.62 in squamous cell carcinoma, adenocarcinoma, and LCNEC, respectively (Table 2). Positive rates and average scores of TrkB and BDNF were highest in LCNEC patients compared to the patients with other histological subtypes. We noted significant correlation coefficient between the scores of TrkB and BDNF in lung cancer using Spearman's rank correlation coefficient (rs=0.580, $P<0.0001$).

Correlations between expression of TrkB and BDNF and clinicopathological factors in NSCLC

The relationship between clinicopathological factors of NSCLC and expression of TrkB and BDNF were shown in Table 1. TrkB and BDNF expression was determined to be positive or negative. Out of all clinicopathological factors, only vascular invasion showed a significant correlation with TrkB (P=0.010) and BDNF (P=0.015) expression. No statistical differences were found between other clinicopathological factors (age, sex, smoking history, histological differentiation, lymphatic invasion, T status, N status, and pathological stage) and TrkB or BDNF expression.

Association between expression of TrkB and disease-free survival and overall survival in NSCLC

Patients were divided into TrkB-positive or -negative groups, and also into BDNF-positive or -negative groups. Kaplan-Meier curves for disease-free survival and overall survival are shown in Figure 2. Statistical analyses were performed using log-rank tests. Patients with TrkB-positive tumors had a significantly poor disease-free survival (P=0.0094) and overall survival (P=0.0019) than those with TrkB-negative tumors, with a median follow-up period of 1470 days. The surgical procedure and adjuvant chemotherapy administered did not differ between patients with TrkB-positive tumors and those with TrkB-negative tumors (Table 1). In histological subgroup analysis, patients with TrkB-positive tumors also had significantly poor overall survival with squamous cell carcinoma (P=0.027) and adenocarcinoma (P=0.0116). The same result was obtained for the subgroup analysis of both the stage I and stage II cases; patients with TrkB-positive tumors had a significantly poor overall survival than those with TrkB-negative tumors (P=0.0065). There were no significant correlations between BDNF expression and disease-free survival and overall survival. Moreover, we tried to identify subgroups with better or poor prognosis by double stratification for TrkB expression and BDNF expression. We divided 102 patients into three groups, double-negative (-), TrkB- or BDNF-positive (+), and double-positive (++). The double-positive group had a significantly poor overall survival and the double-negative group had the best overall survival (P=0.0139; Fig. 2c). The TrkB- or BDNF-positive groups showed medium overall survival among the three groups. Patients were divided into two groups according to the TrkB scores: >3 (higher expression) or ≤ 3 (lower expression). Although there was a tendency towards a worse prognosis in TrkB-higher expression patients, there was no significant correlation.

Univariate and multivariate analysis of prognostic factors in NSCLC patients

Univariate analysis using the Cox proportional hazard model revealed that positive TrkB, lymphatic invasion and stage significantly correlated with disease-free survival and overall

181 survival. Positive BDNF was also correlated with only overall survival in the analysis (Table 3).
182 Multivariate analysis with factors proven to be significant in the univariate analysis revealed
183 that TrkB expression was an independent prognostic factor for disease-free survival (hazard
184 ratio: HR 3.735, 95% confidence interval: C.I. 1.560–11.068, *P*=0.002) and overall survival
185 (HR 4.335, 95% C.I. 1.534–15.963, *P*=0.004) by the Cox hazard model. Lymphatic invasion
186 (HR 2.546, 95% C.I. 1.226–4.984 *P*=0.014) was also a significant independent factor with
187 disease-free survival and overall survival (Table 4).
188
189 **Discussion**
190 Activation of RTKs plays an important role in the progression of malignant tumors, and some
191 mechanisms underlying this activation have been reported previously. Exon 19 deletion or exon
192 21 mutation in the tyrosine kinase domain of epidermal growth factor receptor (EGFR) gene
193 were identified as activating mutations in patients with NSCLC, and EGFR-tyrosine kinase
194 inhibitors (EGFR-TKI) have become key drugs for NSCLC patients harboring EGFR activating
195 mutations [21-23]. Transforming rearrangements of RTK gene, such as echinoderm
196 microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) gene, has
197 also been reported as another mechanism of RTK activation [24]. Overexpression of RTKs and
198 their ligands are also thought to be one of the RTK activating mechanisms [25]. Indeed,
199 overexpression of TrkB and BDNF has been reported in other cancers [9-17]. However, the
200 clinical relevance of TrkB expression in patients with NSCLC remains unclear.
201 There are only a few previous reports of TrkB and BDNF expression in lung cancer [20, 26,
202 27]. The positive rate of TrkB in NSCLC varies from 24% to 86.7% in previous studies [20, 26,
203 27]. Our study showed that 75.5% and 82.4% of NSCLC samples were positive for TrkB and
204 BDNF, respectively, and TrkB and BDNF were highly expressed in lung cancer tissue. Positive
205 rates and average scores of TrkB and BDNF were highest in LCNEC patients compared to the
206 patients with other histological subtypes. High expression levels of TrkB and BDNF might be

involved in neuroendocrine differentiation of LCNEC. We also noted a significant correlation coefficient between TrkB and BDNF scores in lung cancer. Based on the findings, we speculate that TrkB and BDNF might cooperate.

Zhang S *et al* reported that higher TrkB expression in NSCLC was correlated with lymph node metastasis [20], but our study showed no significant correlation. However, we identified a relationship between TrkB and BDNF expression and vascular invasion following examination of clinicopathological factors. Several lines of evidence indicate that activation of the BDNF-TrkB signaling pathway enhances the migratory capability and invasiveness of cancer cells [5-7, 17, 20]. Our results indicate that expression of TrkB and BDNF might promote vascular invasion in lung cancer. Furthermore, it was reported that TrkB and BDNF positively regulated vascular endothelial growth factor (VEGF) expression and tumor-associated angiogenesis [28, 29]. TrkB and BDNF might promote metastasis of tumor cells through vascular invasion and angiogenesis synergistically.

In this study, expression of TrkB was correlated with poor disease-free survival and overall survival in NSCLC; a similar result was obtained through analysis of the histological and stage subgroups. However the case number of each subgroup may be too small to make a definitive conclusion. Furthermore, by double stratification analysis for TrkB expression and BDNF expression, the double-positive group was shown to have significantly poor overall survival and the double-negative group had the best overall survival. The results suggest that the TrkB pathway is activated upon BDNF binding to TrkB receptor. In multivariate analysis, positive TrkB expression was a poor prognostic factor. Our results differ from a previous study that reported TrkB-positive patients with squamous cell lung carcinoma had a better outcome, and there was no correlation between TrkB expression and overall survival of lung cancer patients with adenocarcinoma [26]. A possible explanation of this discrepancy may be that a different method was used to evaluate the expression level of TrkB immunohistochemically, as a standard method has not yet been established. Several factors should be standardized, such as the

antibody used, the definition of positive TrkB staining and so on.

Recently, targeted cancer therapies of RTKs have been developed. EGFR-TKI [21-23, 30, 31], ALK inhibitors [32, 33] and BCL-Abl/KIT inhibitors [34-36] are widely used in clinical settings as standard cancer therapies. These drugs are effective for patients with activating mutations or transforming rearrangements of RTK genes. Furthermore, a large-scale clinical study showed that an EGFR-TKI, erlotinib, was also effective for the patients without EGFR activating mutations [30, 31]. TrkB mutations in non-small cell lung cancer have been reported [37, 38], but they were not driving mutations [39]. However, our previous study reported that a Trk inhibitor can inhibit wild-type TrkB-induced cell migration and proliferation in the presence of BDNF [39]. Taken together with the previous results, the present data suggests that Trk inhibitors are possible candidates for treatment of TrkB-positive NSCLC patients. Further studies are needed to identify the role of the TrkB-BDNF pathway and the effectiveness of Trk inhibitor in lung cancer patients.

In conclusion, we revealed that TrkB is a significant independent poor prognostic factor in NSCLC. Expression of TrkB and BDNF was associated with vascular invasion, and co-expression of TrkB and BDNF was a poor prognostic factor compared with individual expression of either protein. Our results might provide a novel way to explore targeted therapy in lung cancer patients.

Conflict of interest statement

None declared.

Acknowledgement

This study was supported by research fund in Research Institute for Diseases of the Chest.

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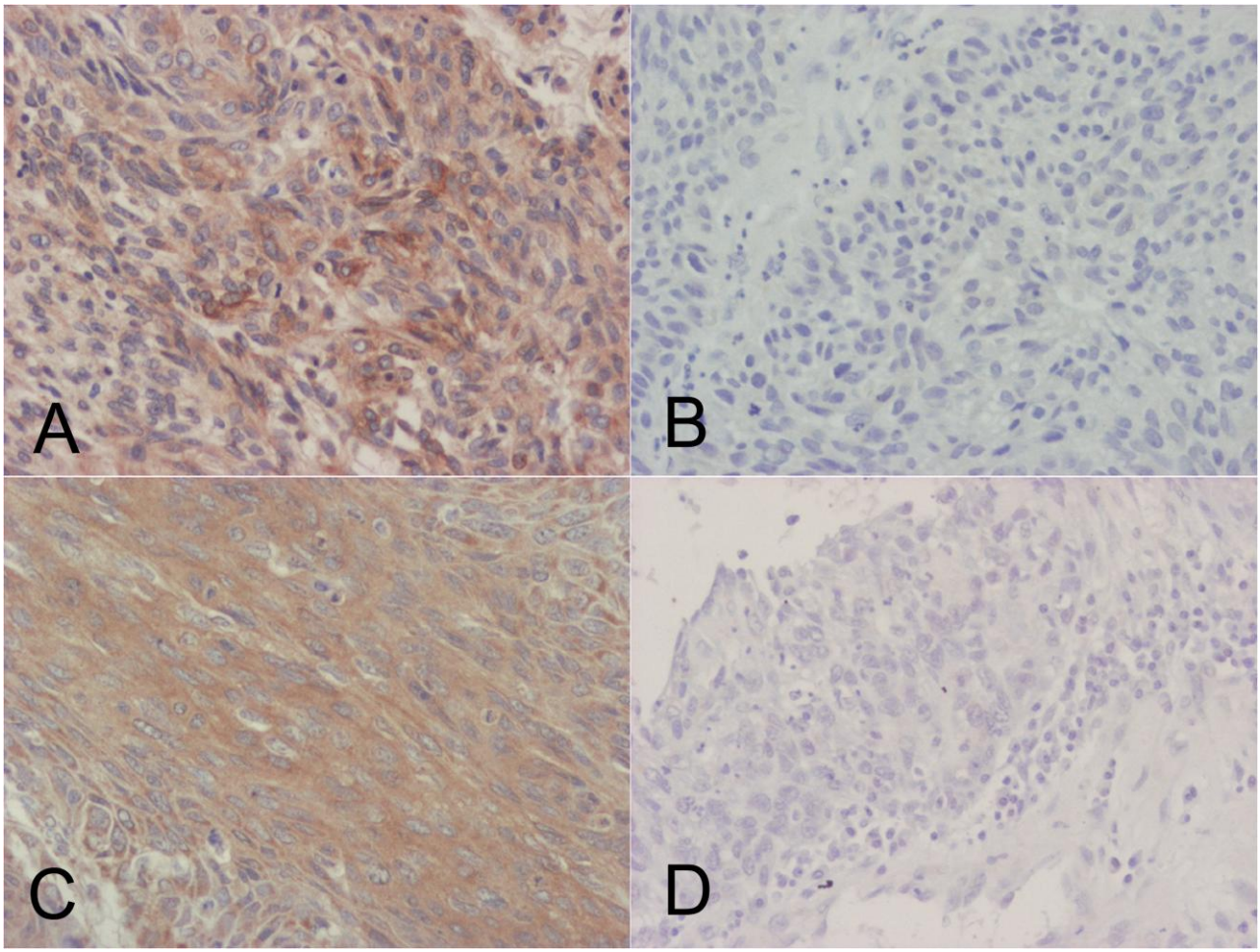
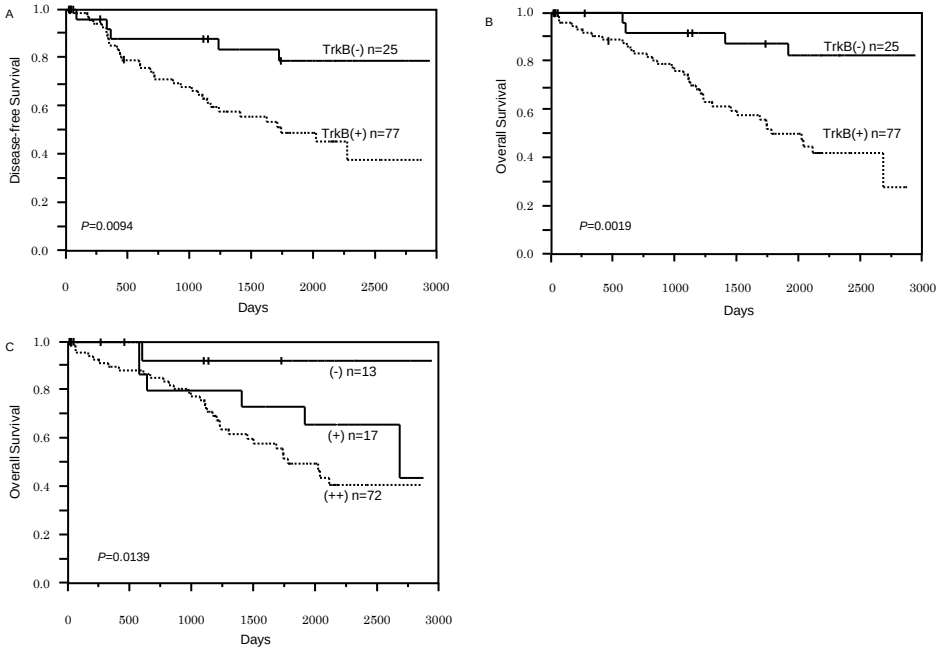
Figure legends

Figure 1. Expression of TrkB and BDNF in squamous cell lung cancer. (A) Positive TrkB staining in tumor cells (200×). (B) Negative TrkB staining in tumor cells (200×). (C) Positive BDNF staining in tumor cells (200×). (D) Negative BDNF staining in tumor cells (200×).



376 **Figure 2. Kaplan-Meier survival curves for NSCLC patients with TrkB-positive or**
377 **TrkB-negative tumors.** Patients with TrkB-positive tumors have poor disease-free survival (A)
378 and overall survival (B) compared to those with TrkB-negative tumors. (C) Patients were
379 divided into three groups according to TrkB expression (positive or negative) and BDNF
380 expression (positive or negative); (-): double negative, (+): BDNF or TrkB positive, (++):
381 double positive. *P*-values calculated with log-rank test are indicated.
382



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384

Table 1. Association between TrkB and BDNF expression and clinicopathological factors in NSCLC.

Factors		Total patients	TrkB-positive	TrkB-negative	*P* value	BDNF-positive	BDNF-negative	*P* value
		n = 102	n = 77	n = 25		n = 84	n =18	
Age	< 70	60	45	15	0.891	51	9	0.405
	≥ 70	42	32	10		33	9	
Sex	Male	72	54	18	0.858	61	11	0.341
	Female	30	23	7		23	7	
Smoking history	Smoker	84	66	18	0.133	71	13	0.236
	Never smoked	18	11	7		13	5	
Differentiation[a]	Well	22	13	9	0.198	15	7	0.076
	Moderately	53	42	11		47	6	
	Poorly	18	14	4		13	5	
Vascular invasion	Absent	60	40	20	0.010*	45	15	0.015*
	Present	42	37	5		39	3	
Lymphatic invasion	Absent	80	61	19	0.736	66	14	0.941
	Present	22	16	6		18	4	
T status	T1	41	29	12	0.350	33	8	0.610
	T2	48	39	9		41	7	
	T3	11	7	4		8	3	
	T4	2	2	0		2	0	
N status	N0	70	51	19	0.224	56	14	0.145
	N1	18	15	3		16	2	
	N2	13	11	2		12	1	
	N3	1	0	1		0	1	
TNM stage	I+II	82	61	21	0.595	67	15	0.723
	III+IV	20	16	4		17	3	
Surgical procedure	Pneumonectomy	6	4	2	0.839	5	1	0.771
	Lobectomy	86	65	21		70	16	
	Segmentectomy	10	8	2		9	1	
Neo-adjuvant therapy	No	99	75	24	0.727	83	16	0.056
	Yes	3	2	1		1	2	
Adjuvant therapy	No	71	53	18	0.764	55	16	0.034*
	Yes	31	24	7		29	2	

*chi-square test , $P < 0.05$

[a] Nine patients with large cell neuroendocrine carcinoma were excluded from the analysis.

Table 2. Expression of TrkB and BDNF in lung cancer defined by histological subtype.

Histological subtype	Total patients n=102	TrkB-positive n (%) Score[a]	BDNF-positive n (%) Score[a]
Squamous cell carcinoma	57	42 (73.7%)	47 (82.5%)
		2.65 ± 0.28	3.37 ± 0.29
Adenocarcinoma	36	27 (75.0%)	28 (77.8%)
		2.39 ± 0.34	2.58 ± 0.33
LCNEC	9	8 (88.9%)	9 (100%)
		3.33 ± 0.62	3.77 ± 0.62

LCNEC = large cell neuroendocrine carcinoma

[a] Score data are mean ± standard error of the mean.

Table 3. Univariate analysis of clinicopathological factors for disease-free survival and overall survival in 102 NSCLC patients by Cox hazard method.

Factors	Disease-free survival			Overall survival		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
TrkB-positive	3.270	1.389 - 9.593	0.005*	4.484	1.786 - 15.040	0.0006*
BDNF-positive	2.512	0.996 - 8.445	0.051	2.730	1.073 - 9.267	0.034*
Age >70	1.018	0.519 - 1.937	0.958	0.994	0.509 - 1.875	0.984
Vascular invasion	1.745	0.920 - 3.335	0.088	1.229	0.650 - 2.294	0.520
Lymphatic invasion	3.012	1.489 - 5.810	0.0029*	2.402	1.170 - 4.646	0.018*
Stage (I+II vs III+IV)	2.740	1.331 - 5.324	0.0075*	2.491	1.212 - 4.823	0.015*

*P <0.05
CI = confidence interval

Table 4. Multivariate analysis of clinicopathological factors for disease-free survival and overall survival in 102 NSCLC patients by Cox hazard method.

Factors	Disease-free survival			Overall survival		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
TrkB-positive	3.735	1.560 - 11.068	0.002*	4.335	1.534 - 15.963	0.004*
BDNF-positive				1.254	0.425 - 4.746	0.703
Lymphatic invasion	3.118	1.481 - 6.251	0.0035*	2.546	1.226 - 4.984	0.014*
Stage (I+II vs III+IV)	1.862	0.880 - 3.727	0.101	2.019	0.977 - 3.933	0.057

*P <0.05
CI = confidence interval