Tropomyosin-related Kinase B Inhibitor Has Potential for Tumor Regression and Relapse Prevention in Pulmonary Large Cell Neuroendocrine Carcinoma

SEIICHI ODATE[1*], HIDEYA ONISHI[1*], KATSUYA NAKAMURA[2], MASAYUKI KOJIMA[3], AKIHIKO UCHIYAMA[4], MASATO KATO[5] and MITSUO KATANO[1]

Departments of [1]Cancer Therapy and Research, and [2]Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [3]Department of Surgery, Japanese Red Cross Fukuoka Hospital, Fukuoka, Japan; [4]Department of Surgery, Kyushu Kosei Nenkin Hospital, Fukuoka, Japan; [5]Department of Surgery, Hamanomachi Hospital, Fukuoka, Japan

Abstract. *Large cell neuroendocrine carcinoma (LCNEC) has an especially poor prognosis, and an effective therapeutic strategy has yet to be established. We have previously shown that the expressions of tropomyosin-related kinase B (TRKB) and brain-derived neurotrophic factor (BDNF) are high in LCNEC and that TRKB/BDNF signaling is involved in the proliferation, tumorigenesis, and invasive nature of LCNEC. Therefore, TRKB/BDNF signaling may offer a potential therapeutic target for LCNEC treatment. In the present study, we evaluated whether the TRKB tyrosine kinase inhibitor, k252a, has effects on tumor regression and relapse prevention on LCNEC, using a murine xenograft model. The LCNEC cell line and NCI-H810 cells were subcutaneously implanted into the flanks or intrathoracically injected into the bilateral pleural cavities of BALB/c nude mice. k252a significantly inhibited tumor volume, expression of matrix metalloproteinases and the formation of pleural dissemination by LCNEC. These results suggest that k252a has potential for tumor regression and relapse prevention in LCNEC. Since many patients with LCNEC suffer through the use of ineffective therapeutic strategies, a clinical trial using the TRKB inhibitor for LCNEC is urgently required.*

*These Authors contributed equally to this work.

Correspondence to: Hideya Onishi, MD, Ph.D., Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel: +81 926426220, Fax: +81 926426221, e-mail: ohnishi@surg1.med.kyushu-u.ac.jp

Key Words: Large cell neuroendocrine carcinoma, TRKB, BDNF, invasion, tumorigenicity, lung cancer.

Lung cancer is the most commonly diagnosed cancer as well as the leading cause of cancer-related mortality in males (1). The prevalence of lung cancer and the resultant mortality are increasing. Of the different types of non-small cell lung cancer (NSCLC), large cell neuroendocrine carcinoma (LCNEC) has especially poor prognosis (2). Currently, the chemotherapy regimen for NSCLC is applied to LCNEC, with limited success. Hence, an effective therapeutic strategy for LCNEC is urgently needed.

Tropomyosin-related kinase B (TRKB) is a member of the TRK family and functions as a receptor tyrosine kinase for brain-derived neurotrophic factor (BDNF). TRKB/BDNF signaling is reported to be a negative prognostic factor for several cancers including neuroblastoma, bladder cancer, pancreatic cancer and ovarian cancer (3-6). Previously, we showed that LCNEC shows high TRKB and BDNF expression compared to other histological types, and that TRKB/BDNF signaling contributes to the proliferation, tumorigenesis, and invasiveness of LCNEC (7). Therefore, TRKB/BDNF signaling is thought to be a therapeutic target for LCNEC. Lestaurtinib (CEP-701; Cephalon, Frazer, PA, USA) is a recently developed small-molecule inhibitor of several receptor tyrosine kinases, and an ATP competitive inhibitor of TRK kinase (8). In phase I clinical trials, lestaurtinib was confirmed to be well-tolerated in patients with neuroblastoma and a dose level sufficient to inhibit TRKB activity was established (9). In addition, it has been shown, in a mouse neuroblastoma xenograft model, that lestaurtinib enhances the efficacy of conventional chemotherapy by inhibiting the TRK/BDNF pathway (10). With a view to developing a new therapeutic strategy for refractory LCNEC, in the present study we evaluated whether the TRKB tyrosine kinase inhibitor k252a shows effects of tumor regression and relapse prevention on LCNEC in a murine xenograft model.

Materials and Methods

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 experiments were approved by the Ethics Committee of Kyushu University (Inspection No. A25-027-0). The LCNEC cell line and NCI-H810 cells were subcutaneously implanted into the flanks (5×10^6 cells in 100 μl of phosphate buffered saline; PBS per mouse) of nude mice (n=6 in each case). After the tumor reached 300 mm^3 in size, k252a (9 μg in 200 μl of saline; Alamone Labs, Jerusalem, Israel) was administrated intraperitoneally twice a week. In another experiment, to create a model of LCNEC pleural dissemination, NCI-H810 cells were intrathoracically injected into the bilateral pleural cavities (5×10^6 cells in 100 μl of PBS per mouse) of nude mice (n=5 in each case). Two weeks after tumor injection, k252a (9 μg in 200 μl of saline) or an equivalent dose of saline (controls) were administered intraperitoneally once a week. Forty-five days after tumor injection, mice were sacrificed and the tumor number and volume were evaluated. The tumor volume was calculated by the following formula: length × $(width)^2\times$ 0.5 (mm^3). k252a dosage was determined in reference to the previous report (11).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick- end labeling (TUNEL) assay. Detection of apoptotic cells within xenografted tumors was performed using an In Situ Apoptosis Detection Kit (Takara Bio, Tokyo, Japan) according to the manufacturer's protocol.

Fluorescence immunohistochemistry. Primary antibodies used were against matrix metalloproteinase (MMP)-2 (1:100, sc-10736; Santa Cruz Biotechnology, Dallas, TX, USA) and MMP-9 (1:100, sc-6840; Santa Cruz Biotechnology). Secondary antibodies used were Alexa 488-conjugated chicken anti-rabbit (1:1000; Life Technologies, Grand Island, NY, USA) and Alexa 594-conjugated rabbit anti-goat (1:1000; Life Technologies). Cells were counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich, St. Louis, MO, USA). Samples were semi-quantitatively analyzed by fluorescence microscopy (Axio Imager; Carl Zeiss, Oberkochen, Germany) (12).

Statistical analysis. All data are represented as mean±SD. A χ^2 test was applied to analyze pleural dissemination model. Student's *t*-test was used for the comparison of mean values between two groups. Overall survival was calculated by the Kaplan–Meier method and analyzed by the log-rank test. A *p*-value of <0.05 was considered significant.

Results

The TRKB tyrosine kinase inhibitor, k252a, reduces tumor progression in vivo. Firstly in order to test if k252a has potential in therapeutic treatment for LCNEC, either k252a or an equivalent volume of saline were administered intraperitoneally twice a week, once the volume of the xenograft tumor had reached 300 mm^3. The mean tumor volume in mice that had been administered k252a was significantly lower than in mice administered saline alone (Figure 1A). Apoptotic cells were more prevalent in mice administered k252a compared with mice administered saline alone (Figure 1B). Previously, we revealed that MMP-2 and MMP-9, which are type IV collagenases, are located downstream of TRKB/BDNF signaling (7). Therefore, we next evaluated the expression levels of MMP-2 and MMP-9. Consistent with our previous results (7), the expression levels of MMP-2 and MMP-9 in tumors from k252a-injected mice were significantly lower compared with those of control mice, potentially reflecting reduced invasiveness of the k252a injected tumors (Figure 1C and D).

Table I. *The dissemination in mice of tumors injected intrathoracically*.

Dissemination	Saline	k252a	*p*-Value
+	5	2	
–	0	3	0.0384*

p-Value was calculated by chi-square test. *Statistically significant.

k252a inhibits tumorigenicity and shows potential for prevention of relapse in pleural dissemination model in vivo. Next, to evaluate if k252a inhibits tumorigenicity and has potential as a drug for prevention of relapse of LCNEC, either k252a or an equivalent volume of saline was administered intraperitoneally once a week starting two weeks after the tumor-initiating injection into the bilateral thoracic cavity. At 45 days after the initial injection, mice were sacrificed and tumor size and number within the thoracic cavity were evaluated. Tumors within the thoracic cavity were seen in two out of the five mice that had been injected with k252a, while tumors were seen in all five of the control mice (Table I). Both the number and volume of the tumors in the k252a-injected mice were significantly lower than those of the control mice (Figure 2A-C). These results suggest that k252a can prevent relapse and inhibit thoracic dissemination in LCNEC.

Discussion

Despite the poor prognosis of LCNEC, no consensus on its therapy has been reached. One of the mechanisms for the aggressiveness of LCNEC may be its high capacity for tumorigenesis, proliferation and invasiveness. If these attributes could be inhibited, the prognosis of LCNEC should improve. Therefore, in this study, we established specific mouse models; by injecting tumor cells into the flank subcutaneously and also intrathoracically in order to evaluate the effect of the TRKB tyrosine kinase inhibitor, k252a. Consistent with previous reports that TRKB/BDNF signaling contributes to both anchorage-dependent and -independent growth (7), the tumors of mice injected with k252a were

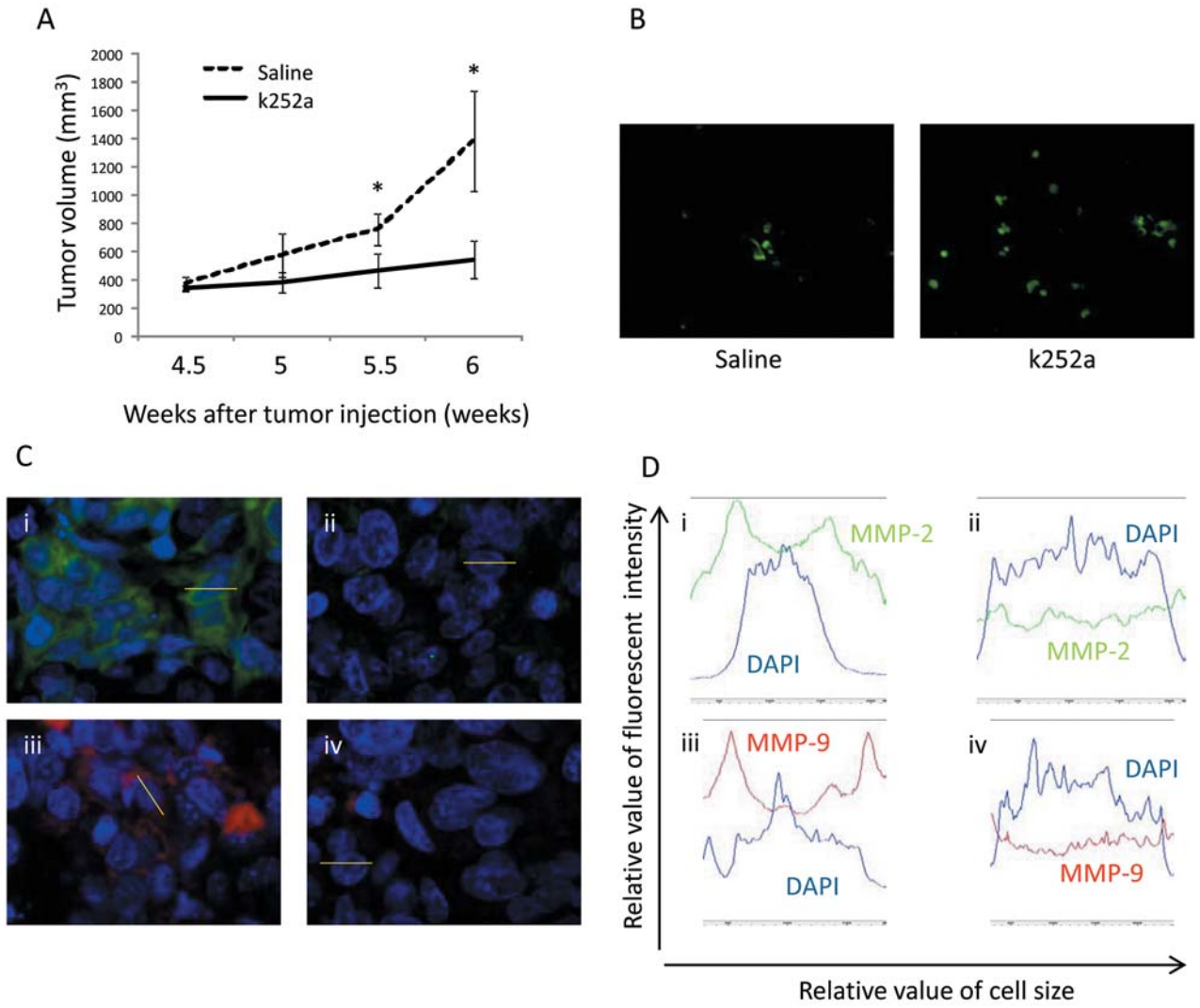

Figure 1. *The TRKB tyrosine kinase inhibitor k252a reduces tumorigenicity in vivo. NCI-H810 cells were subcutaneously injected into mice. k252a (9 μg/body) was intraperitoneally injected twice a week, after the tumor volume reached 300 mm^3 in size. A: Tumor volume was investigated on the indicated day. The graph shows mean±SD. *p<0.05. B: Apoptotic tumor cells from the xenograft model were examined by Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick- end labeling (TUNEL) staining. Apoptotic tumor cells were labeled with fluorescein isothiocyanate (FITC). Representative images are shown. The original magnification was ×400. C: Representative images of immunofluorescent staining for matrix metalloproteinase (MMP)-2 (i, ii; green), MMP-9 (iii, iv; red) with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (blue) in the control group (i, iii) and k252a-treated group (ii, iv). The original magnification was ×630. D: MMP-2 (i, ii; green) and MMP-9 (iii, iv; red) expressions of a single cell (yellow line in C) in the control group (i, iii) and k252a group (ii, iv) level was semi-quantitatively estimated by fluorescent microscopy.*

significantly reduced in volume. On the other hand, MMPs are reported to have a pivotal role in invasiveness due to their ability to degrade type IV collagen (13). We also showed that k252a reduced the expression of MMPs *in vivo* using a semi-quantitative fluorescence immunohistochemical staining method (12). These results suggest that k252a could be used as a therapeutical drug for LCNEC.

We performed experiments using an intrathoracically tumor-injected model for the evaluation of adjuvant chemotherapy with k252a. In this experiment, three out of five mice that had been injected with k252a did not experience dissemination in the thoracic cavity. We think that this result suggests that k252a may also be used as an adjuvant chemotherapy drug for LCNEC.

As shown in our previous report, all patients with LCNEC showed high expressions of TRKB and BDNF, therefore survival analysis between the low-expression and high-expression groups could not be performed (7). On the other

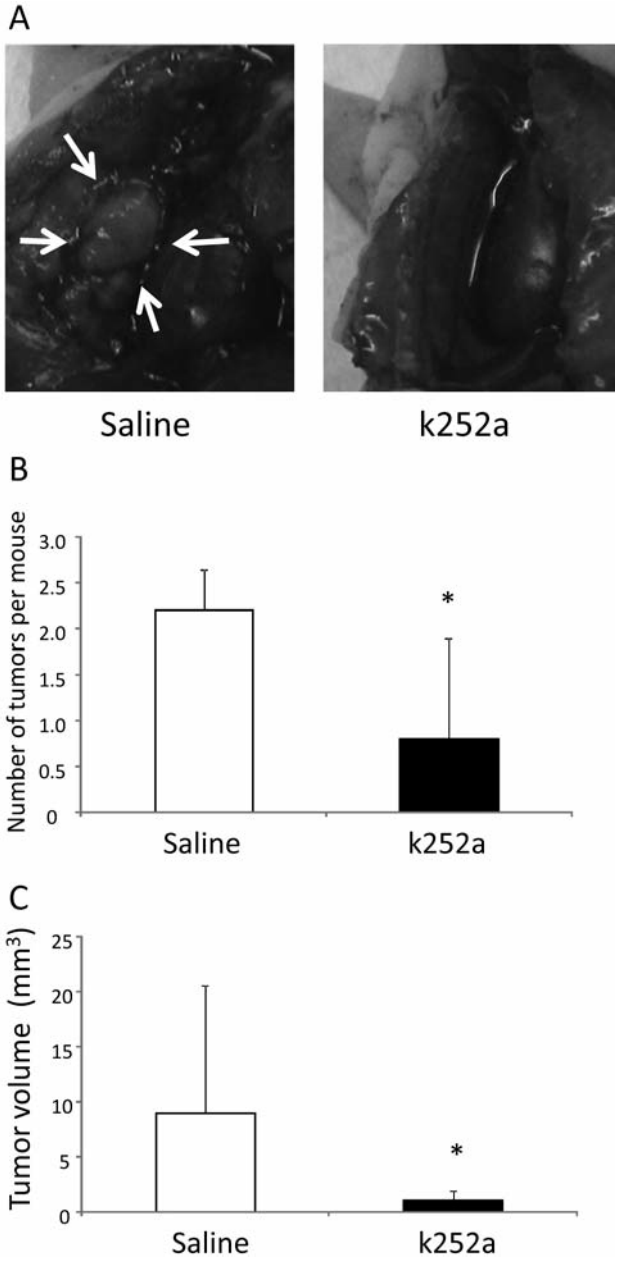

Figure 2. *k252a inhibits the formation of pleural dissemination in large cell neuroendocrine carcinoma (LCNEC). NCI-H810 cells were intrathoracically injected into the bilateral pleural cavities of nude mice. Forty-five days after tumor cell injection, mice were sacrificed and tumor number and size were evaluated. A: Representative images of the right thoracic cavity of a k252a injected mouse (right panel) and a control mouse (left panel). Arrows show tumor formed. B: The number of tumors within the thoracic cavity was counted. C: The volume of thoracic cavity tumors was evaluated. The graph shows the mean±SD. *p<0.05.*

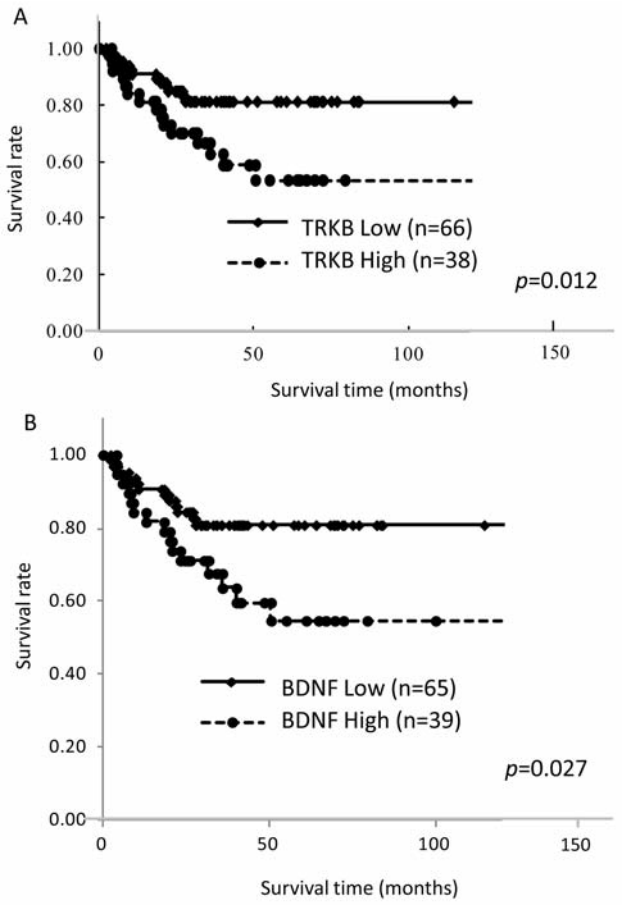

Figure 3. *After cancer tissues from patients with non-small cell lung cancer (NSCLC) who underwent surgical resection at Hamanomachi Hospital and Kyushu Kosei Nenkin Hospital were stained for tropomyosin-related kinase B (TRKB) and brain-derived neurotrophic factor (BDNF) immunohistochemically, total score, calculated by multiplying intensity score and percentage score, was estimated as reported in our previous report (7). The patients were then divided into two groups by total score on the basis of the mean value. Overall survival of patients with low and high TRKB expression (A) and BDNF expression (B) was calculated by the Kaplan–Meier method and analyzed by the log-rank test.*

hand, patients with NSCLC with a high expression of TRKB or BDNF had a poor prognosis compared to those expressing low levels of TRKB or BDNF (Figure 3), suggesting that TRKB/BDNF signaling may be a potential therapeutic target in other histological types where TRKB/BDNF signaling is activated, as well as LCNEC, and that k252a may be effective against several types of lung cancer.

Clinically, lestaurtinib has been used as an inhibitor of TRKB instead of k252a. However, clinical trials using lestaurtinib have been performed only in patients with neuroblastoma. In the present study, using a murine xenograft model, we have for the first time provided evidence that the TRKB tyrosine kinase inhibitor, k252a, produces effects of tumor regression and relapse prevention

in LCNEC. Since many patients with LCNEC suffer through ineffective therapeutic strategies, a clinical trial for LCNEC using the TRKB inhibitor is needed.

Conflicts of Interest

All Authors declare no conflicts of interest.

Acknowledgements

This study was supported by JSPS KAKENHI grant number 23592065. We thank Ms. Kaori Nomiyama for skillful technical assistance.

References

1 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin *61*: 69-90, 2011.

2 Asamura H, Kameya T, Matsuno Y, Noguchi M, Tada H, Ishikawa Y, Yokose T, Jiang SX, Inoue T, Nakagawa K, Tajima K and Nagai K: Neuroendocrine neoplasms of the lung: A prognostic spectrum. J Clin Oncol *24*: 70-76, 2006.

3 Matsumoto K, Wada RK, Yamashiro JM, Kaplan DR and Thiele CJ: Expression of brain-derived neurotrophic factor and pl4S TRKB affects survival, differentiation, and invasiveness of human neuroblastoma cells. Cancer Res *55*: 1798-1806, 1995.

4 Dionne CA, Camoratto AM, Jani JP, Emerson E, Neff N, Vaught JL, Murakata C, Djakiew D, Lamb J, Bova S, George D and Isaacs JT: Cell cycle-independent death of prostate adenocarcinoma is induced by the TRK tyrosine kinase inhibitor CEP-751 (KT6587). Clin Cancer Res *4*: 1887-1898, 1998.

5 Miknyoczki SJ, Lang D, Huang L, Klein-Szanto AJ, Dionne CA and Ruggeri BA: Neurotrophins and TRK receptors in human pancreatic ductal adenocarcinoma: Expression patterns and effects on *in vitro* invasive behavior. Int J Cancer *81*: 417-427, 1999.

6 Au CW, Siu MK, Liao X, Wong ES, Ngan HY, Tam KF, Chan DC, Chan QK and Cheung AN: Tyrosine kinase B receptor and BDNF expression in ovarian cancers – Effect on cell migration, angiogenesis and clinical outcome. Cancer Lett *281*: 151-161, 2009.

7 Odate S, Nakamura K, Onishi H, Kojima M, Uchiyama A, Nakano K, Kato M, Tanaka M and Katano M: TRKB/BDNF signaling pathway is a potential therapeutic target for pulmonary large cell neuroendocrine carcinoma. Lung Cancer *79*: 205-214, 2013.

8 Miknyoczki SJ, Dionne CA, Klein-Szanto AJ and Ruggeri BA: The novel TRK receptor tyrosine kinase inhibitor CEP-701 (KT-5555) exhibits antitumor efficacy against human pancreatic carcinoma (Panc1) xenograft growth and *in vivo* invasiveness. Ann NY Acad Sci *880*: 252-262, 1999.

9 Minturn JE, Evans AE, Villablanca JG, Yanik GA, Park JR, Shusterman S, Groshen S, Hellriegel ET, Bensen-Kennedy D, Matthay KK, Brodeur GM and Maris JM: Phase I trial of lestaurtinib for children with refractory neuroblastoma: A new approaches to neuroblastoma therapy consortium study. Cancer Chemother Pharmacol *68*: 1057-1065, 2011.

10 Iyer R, Evans AE, Qi X, Ho R, Minturn JE, Zhao H, Balamuth N, Maris JM and Brodeur GM: Lestaurtinib enhances the antitumor efficacy of chemotherapy in murine xenograft models of neuroblastoma. Clin Cancer Res *16*: 1478-1485, 2010.

11 Kawamura N, Kawamura K, Manabe M and Tanaka T: Inhibition of brain-derived neurotrophic factor/tyrosine kinase B signaling suppresses choriocarcinoma cell growth. Endocrinology *151*: 3006-3014, 2010.

12 Kai M, Onishi H, Souzaki M, Tanaka H, Kubo M, Tanaka M and Katano M: Semi-quantitative evaluation of CD44(+)/CD24(–) tumor cell distribution in breast cancer tissue using a newly developed fluorescence immunohistochemical staining method. Cancer Sci *102*: 2132-2138, 2011.

13 Nelson AR, Fingleton B, Rothenberg ML and Matrisian LM: Matrix metalloproteinases: Biologic activity and clinical implications. Int J Oncol *18*: 1135-1149, 2000.

Received June 11, 2013
Revised July 1, 2013
Accepted July 3, 2013