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TrkB/BDNF Signaling Could Be a New Therapeutic Target for Pancreatic Cancer

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Abstract. *Background/Aim: Tropomyosin-related kinase B (TrkB)/brain-derived neurotrophic factor (BDNF) signaling plays a role in inducing malignant phenotypes in several aggressive types of cancers. To create a conclusive therapy targeting TrkB/BDNF signaling in solid refractory cancers, the biological significance of TrkB/BDNF signaling was analyzed in pancreatic ductal adenocarcinoma (PDAC) cells. Materials and Methods: Three PDAC cell lines were used as target cells to investigate proliferation and invasiveness. Small interfering RNA (siRNA) and the TrkB tyrosine kinase inhibitor k252a were used as TrkB/BDNF signaling inhibitors. Results: All PDAC cell lines expressed TrkB and BDNF. When TrkB and BDNF were inhibited by siRNA or k252a, the invasiveness of PANC-1 and SUIT-2 cells significantly decreased. When TrkB was inhibited by siRNA or k252a, proliferation was significantly inhibited in PDAC cells. Conclusion: TrkB/BDNF signaling may be a new therapeutic target for PDAC. Therapies targeting TrkB/BDNF signaling may be a conclusive cancer therapy for refractory solid cancer.*

Pancreatic ductal adenocarcinoma (PDAC) is a refractory cancer phenotype (1). Surgical resection is the only curative effective therapy; however, its indication is extremely low because the disease has typically already advanced at the time of diagnosis. Meanwhile, the types and effects of chemotherapeutic agents remain limited. Currently, there are no effective treatments for advanced and recurrent diseases. Therefore, the development of new therapeutic strategies is urgently required.

Tropomyosin-related kinase B (TrkB) is a member of the Trk family and acts as a receptor tyrosine kinase for brain-derived neurotrophic factor (BDNF), a member of the nerve growth factor family. In normal cells, TrkB/BDNF signaling plays a role in the development of the nervous system (2, 3). However, in cancer cells, it has been shown that TrkB/BDNF signaling plays a role in inducing the aggressive phenotype of several cancers and is a factor of poor prognosis (4-6). TrkB/BDNF signaling is expected to be a promising therapeutic target for refractory solid cancers.

In this study, we investigated the biological significance of TrkB/BDNF signaling in PDAC to develop an effective therapy for PDAC.

Materials and Methods

Cell culture and reagents. Three human PDAC cell lines (ASPC-1, SUIT-2, and PANC-1) were maintained in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS; Life Technologies, Grand Island, NY, USA) and antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin; Meijiseika, Tokyo, Japan).

Western blot analysis. Western blotting was performed as previously described (7). Protein samples (50 μg) were separated by electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to Protran nitrocellulose membranes (Whatman, Dassel, Germany). The protein-transferred membranes were incubated overnight at 4˚C with primary antibodies for TrkB (1:200, sc-8316, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and BDNF (1:200, sc-546, Santa Cruz Biotechnology). The membranes were then incubated with peroxidase-linked secondary antibodies (Amersham Biosciences, Piscataway, NJ, USA) for 1 h at room temperature. The antibody for α-tubulin (1:1,000, Sigma-Aldrich, St. Louis, MO, USA) was used as a protein loading control.

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Key Words: TrkB, BDNF, pancreatic cancer, proliferation, invasion.

RNA interference. ON-TARGETplus™ SMARTpool small interfering RNA (siRNA) targeting TrkB (L-003160), BDNF (L-017626), and negative control siRNA (ON-TARGETplus™ Control non-targeting siRNA, D-001810) were purchased from Dharmacon (Lafayette, CO, USA). Cells (0.2×10^6 cells/well) seeded in six-well plates were transfected with 100 nM siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were used for experiments at 2 days after transfection.

Invasion assay. Invasion assay was performed with a Matrigel-coated Transwell insert as previously described (8). Briefly, cells (2×10^5) were placed in the upper chamber and incubated for 18 h. The cells that migrated to the lower side of the filter were fixed and stained with Diff-Quik reagent (Sysmex, Kobe, Japan) and then counted under a light microscope (Nikon Eclipse TE 300, Nikon, Tokyo).

Cell proliferation assay. All PDAC cell lines were seeded into 96-well plates at 5,000 cells/well and cultured for 16 h. Cell proliferation was assessed by absorbance (Biotrak visible plate reader, Amersham Biosciences) at 492 nm (reference wavelength 620 nm) using Cell Count Reagent SF (Nacalai Tesque). In some experiments, recombinant BDNF (Peprotech, Rocky Hill, NJ, USA) and TrkB tyrosine kinase inhibitor: K252a (Alomone Labs, Jerusalem, Israel) were added to the culture.

Statistical analyses. The data are presented as the mean±standard deviation (SD). Calculations were carried out using JMP 12.0 software (SAS Institute, Cary, NC, USA) or Microsoft Excel software (Microsoft, Redmond, WA, USA). Student's *t*-test was used to compare continuous variables between pairs of groups. A *p*-value <0.05 was considered significant.

Results

TrkB/BDNF signaling contributes to the invasiveness of PDAC cells. First, the expression of TrkB and BDNF in PDAC cells was confirmed by western blotting. All PDAC cell lines expressed TrkB and BDNF (Figure 1). Next, we investigated whether TrkB/BDNF signaling is involved in the invasiveness of PDAC cells. When TrkB and BDNF were inhibited by siRNA, the invasiveness of PANC-1 and SUIT-2 cells significantly decreased (Figure 2A). When TrkB/BDNF signaling was inhibited by k252a, which is an inhibitor of TrkB tyrosine kinase, the invasiveness of PANC-1 and SUIT-2 cells also significantly decreased (Figure 2B). These results suggest that TrkB/BDNF signaling may contribute to the invasiveness of PDAC cells. However, even if TrkB/BDNF signaling was stimulated by recombinant BDNF, there was no significant change in invasiveness (Figure 2C).

TrkB/BDNF signaling contributes to the proliferation of PDAC cells. We investigated whether TrkB/BDNF signaling is involved in PDAC cell proliferation. When TrkB was inhibited by siRNA, proliferation of all three PDAC cells significantly decreased, while inhibition of BDNF by siRNA did not affect the proliferation of PDAC cells (Figure 3A). When TrkB/BDNF signaling was inhibited by k252a, the proliferation of PANC-1 cells also significantly decreased (Figure 3B). However, even if TrkB/BDNF signaling was stimulated by recombinant BDNF, there was no significant change in invasiveness (Figure 3C). These results suggest that TrkB/BDNF signaling may contribute to the proliferation of PDAC cells.

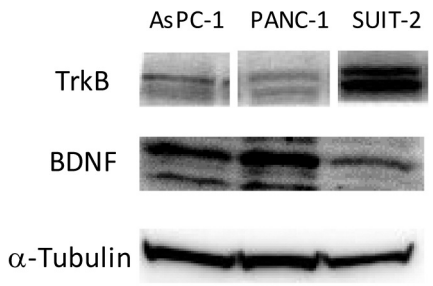

Figure 1. *TrkB and BDNF are expressed in all three cell lines used in the experiments. The protein expression of TrkB and BDNF was examined by western blot analysis. TrkB: Tropomyosin-related kinase B; BDNF: brain-derived neurotrophic factor.*

Discussion

In our study, recombinant BDNF did not affect the proliferation and invasiveness of PDAC cells. We believe that PDAC cells secrete BDNF, and this autocrine BDNF may be sufficient to activate TrkB/BDNF signaling. Among all three PDAC cell lines, it was difficult to decrease the invasiveness of AsPC-1 cells. It is also expected, that antitumor agents are not effective in all patients with PDAC. It is important to understand the differences in AsPC-1 cells compared to the other two cell lines. For example, in the small cell lung cancer (SCLC) line, comprising SBC-5 cells, Hedgehog (Hh) signaling is inversely correlated with TrkB/BDNF signaling, and inhibition of Hh signaling leads to the activation of TrkB/BDNF signaling to induce malignant phenotypes (9). Some factors or signaling pathways may contribute to this discrepancy. For example, with respect to the cancer microenvironment, which has received much attention in current research, TrkB/BDNF signaling is suggested to correlate with hypoxia-inducible factor 1α, which is an important transcriptional factor under hypoxic conditions (10). Research should proceed in this direction.

Trk inhibitors are under investigation in clinical trials for TRK fusion-positive solid tumors. For example, a clinical trial on larotrectinib (LOXO-101), a selective pan-TRK inhibitor, is ongoing for TRK fusion-positive solid tumors,

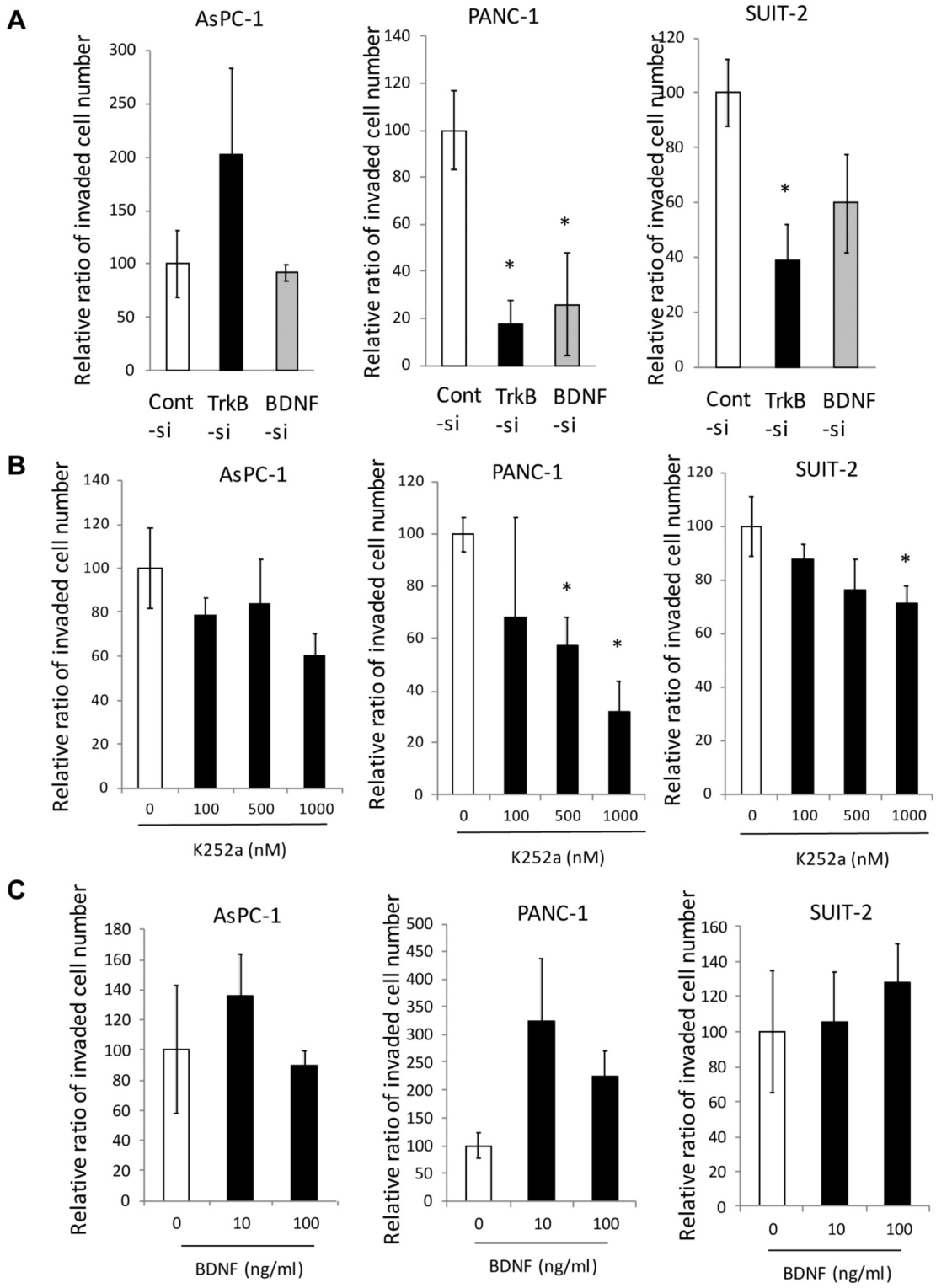

Figure 2. *TrkB/BDNF signaling contributes to the invasiveness of PDAC cells. A: The invasive abilities of PDAC cells transfected with TrkB siRNA or BDNF siRNA were estimated using a Matrigel invasion assay. B: The invasive abilities of PDAC cells treated with k252a at the indicated concentrations were estimated using a Matrigel invasion assay. C: The invasive abilities of PDAC cells treated with recombinant BDNF at the indicated concentrations were estimated using a Matrigel invasion assay. In the invasion assay, all cells that had migrated from the upper to the lower side of the filter were counted. Data are presented as means±standard deviations. *Significantly different at p<0.05. TrkB: Tropomyosin-related kinase B; BDNF: brain-derived neurotrophic factor; PDAC: pancreatic ductal adenocarcinoma; siRNA: small interfering RNA.*

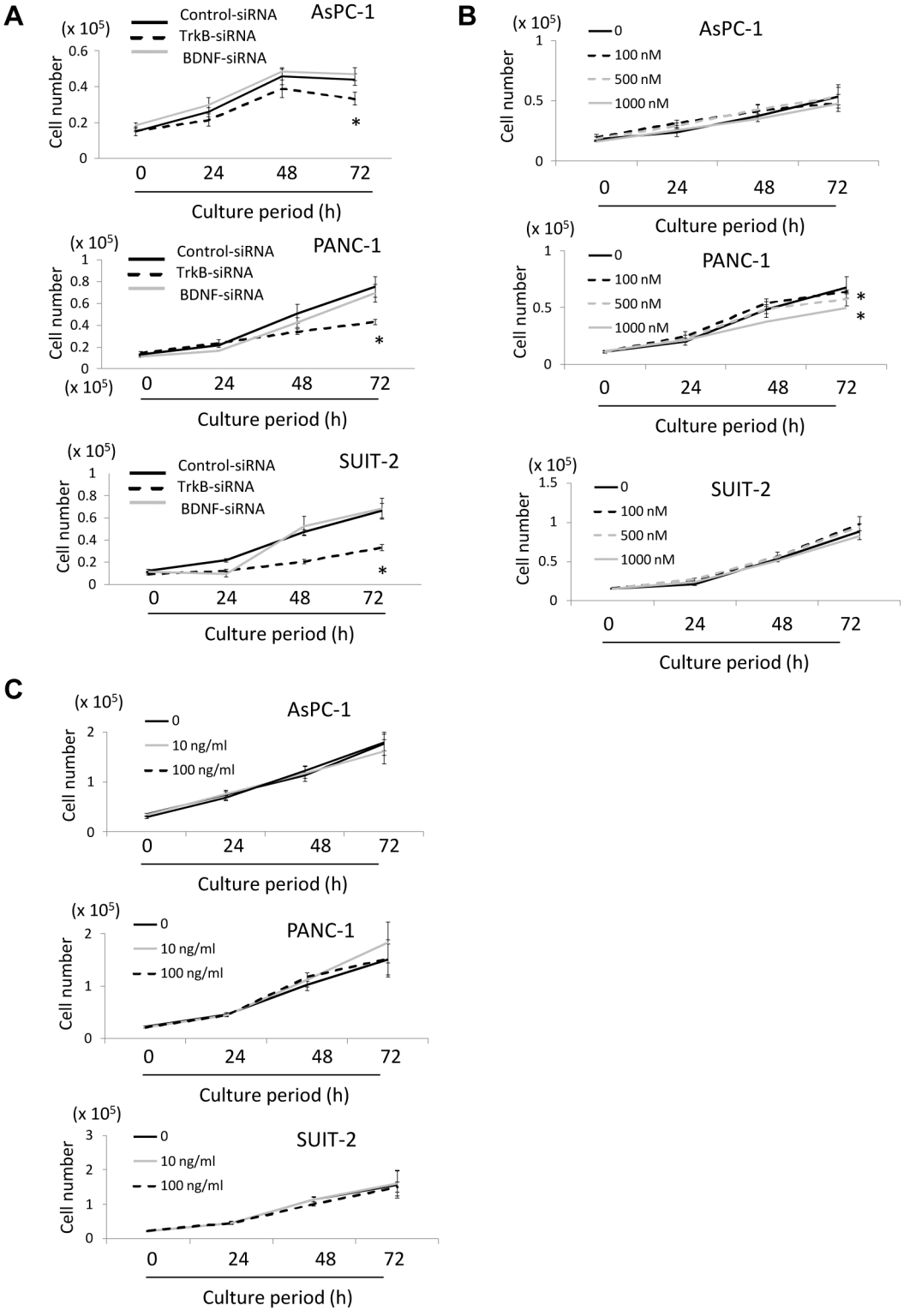

Figure 3. *TrkB/BDNF signaling contributes to the proliferation of PDAC cells. A: Cell numbers at the indicated days among PDAC cells transfected with TrkB siRNA or BDNF siRNA were counted using light microscopy. B: Cell numbers among PDAC cells treated with k252a at the indicated concentrations were counted using light microscopy. C: Cell numbers among PDAC cells treated with recombinant BDNF at the indicated concentrations were counted using light microscopy. Data are presented as means±standard deviations. *Significantly different at p<0.05. TrkB: Tropomyosin-related kinase B; BDNF: brain-derived neurotrophic factor; PDAC: pancreatic ductal adenocarcinoma; siRNA: small interfering RNA.*

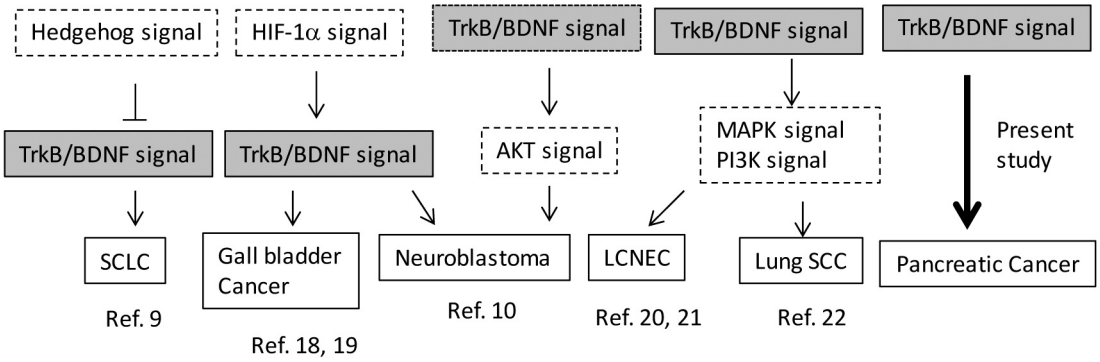

Figure 4. *Schematic results of our studies regarding TrkB/BDNF signaling. Therapies targeting TrkB/BDNF signaling may be a conclusive cancer therapy against refractory solid cancer. TrkB: Tropomyosin-related kinase B; BDNF: brain-derived neurotrophic factor.*

and promising results have been reported (11-13). ONO-7579, a second-generation pan-TRK inhibitor, is expected to be a potent and selective agent that is orally administered (14). Although a hopeful result for PDAC has not yet been obtained, the information obtained in the present study will help improve the effectiveness of Trk inhibitors for PDAC. We believe that the drawback is that the Trk inhibitor is not a selective TrkB inhibitor. Among the Trk family, some studies have shown that TrkA acts as a tumor suppressor in neuroblastoma and colon cancer (15) and that TrkC is a good prognostic factor for medulloblastoma and colon cancer (16, 17). The development of a selective TrkB inhibitor may be difficult because its molecular structure is similar to the proteins of the Trk family, which must be considered.

Figure 4 shows our previous findings on TrkB/BDNF signaling in SCLC (9), gallbladder cancer (18, 19), neuroblastoma (10), large cell neuroendocrine tumors (20, 21), and lung squamous cell carcinoma (22). Studies that target TrkB/BDNF signaling are ongoing in the context of other types of cancers, such as brain glioblastoma and oral squamous cell carcinoma (23, 24).

In the present study, we revealed the possibility that TrkB/BDNF signaling may be a new therapeutic target for PDAC. In conclusion, therapies targeting TrkB/BDNF signaling may be a conclusive cancer therapy for refractory solid cancer.

Conflicts of Interest

The Authors declare no financial or commercial conflicts of interest regarding this study.

Authors' Contributions

Yasuhiro Oyama was involved in the analysis of all experiments. Shinjiro Nagao, Lin Na and Masayo Umebayashi were involved in the acquisition and analysis of data. Kosuke Yanai and Akio Yamasaki were involved in the gene transfection. Katsuya Nakamura, Shuntaro Nagai and Akiko Fujimura were involved in the interpretation of data. Kazunori Nakayama, Takashi Morisaki and Hideya Onishi were involved in the design of the work.

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References

1 Siegel RL, Miller KD and Jemal A: Cancer statistics, 2019. CA Cancer J Clin *69(1)*: 7-34, 2019. PMID: 30620402. DOI: 10.3322/caac.21551

2 Klein R, Parada LF, Coulier F and Barbacid M: trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. EMBO J *8(12)*: 3701-3709, 1989. PMID: 2555172

3 Klein R, Smeyne RJ, Wurst W, Long LK, Auerbach BA, Joyner AL and Barbacid M: Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. Cell *75(1)*: 113-122, 1993. PMID: 8402890.

4 Han L, Zhang Z, Qin W and Sun W: Neurotrophic receptor TrkB: Is it a predictor of poor prognosis for carcinoma patients? Med Hypotheses *68(2)*: 407-409, 2007. PMID: 17008023. DOI: 10.1016/j.mehy.2006.05.038

5 Eggert A, Grotzer MA, Ikegaki N, Zhao H, Cnaan A, Brodeur GM and Evans AE: Expression of the neurotrophin receptor TrkB is associated with unfavorable outcome in Wilms' tumor. J Clin Oncol *19(3)*: 689-696, 2001. PMID: 11157019. DOI: 10.1200/JCO.2001.19.3.689

6 Tanaka K, Mohri Y, Nishioka J, Kobayashi M, Ohi M, Miki C, Tonouchi H, Nobori T and Kusunoki M: Neurotrophic receptor, tropomyosin-related kinase B as an independent prognostic marker in gastric cancer patients. J Surg Oncol *99(5)*: 307-310, 2009. PMID: 19170129. DOI: 10.1002/jso.21232

7 Onishi H, Yamasaki A, Nakamura K, Ichimiya S, Yanai K, Umebayashi M, Nagai S and Morisaki T: Liprin-α4 as a new therapeutic target for SCLC as an upstream mediator of HIF1α. Anticancer Res *39(3)*: 1179-1184, 2019. PMID: 30842147. DOI: 10.21873/anticanres.13227

8 Onishi H, Ichimiya S, Yanai K, Umebayashi M, Nakamura K, Yamasaki A, Imaizumi A, Nagai S, Murahashi M, Ogata H and Morisaki T: RBPJ and MAML3: Potential therapeutic targets for small cell lung cancer. Anticancer Res *38(8)*: 4543-4547, 2018. PMID: 30061220. DOI: 10.21873/anticanres.12758

9 Onishi H, Nakamura K, Nagai S, Yanai K, Yamasaki A, Kawamoto M, Imaizumi A and Morisaki T: Hedgehog inhibition upregulates TRK expression to antagonize tumor suppression in small cell lung cancer cells. Anticancer Res *37(9)*: 4987-4992, 2017. PMID: 28870922. DOI: 10.21873/anticanres.11910

10 Nakamura K, Martin KC, Jackson JK, Beppu K, Woo CW and Thiele CJ: Brain-derived neurotrophic factor activation of TrkB induces vascular endothelial growth factor expression via hypoxia-inducible factor-1alpha in neuroblastoma cells. Cancer Res *66(8)*: 4249-4255, 2006. PMID: 16618748. DOI: 10.1158/0008-5472.CAN-05-2789

11 Drilon A, Siena S, Ou SI, Patel M, Ahn MJ, Lee J, Bauer TM, Farago AF, Wheler JJ, Liu SV, Doebele R, Giannetta L, Cerea G, Marrapese G, Schirru M, Amatu A, Bencardino K, Palmeri L, Sartore-Bianchi A, Vanzulli A, Cresta S, Damian S, Duca M, Ardini E, Li G, Christiansen J, Kowalski K, Johnson AD, Patel R, Luo D, Chow-Maneval E, Hornby Z, Multani PS, Shaw AT and De Braud FG: Safety and antitumor activity of the multitargeted Pan-TRK, ROS1, and ALK inhibitor entrectinib: Combined results from two phase I trials (ALKA-372-001 and STARTRK-1). Cancer Discov *7(4)*: 400-409, 2017. PMID: 28183697. DOI: 10.1158/2159-8290.CD-16-1237

12 Italiano A, Nanda S, Briggs A, Garcia-Foncillas J, Lassen U, Vassal G, Kummar S, van Tilburg CM, Hong DS, Laetsch TW, Keating K, Reeves JA, Fellous M, Childs BH, Drilon A and Hyman DM: Larotrectinib versus prior therapies in tropomyosin receptor kinase fusion cancer: an intra-patient comparative analysis. Cancers (Basel) *12(11)*: 3246, 2020. PMID: 33158040. DOI: 10.3390/cancers12113246

13 Hong DS, DuBois SG, Kummar S, Farago AF, Albert CM, Rohrberg KS, van Tilburg CM, Nagasubramanian R, Berlin JD, Federman N, Mascarenhas L, Geoerger B, Dowlati A, Pappo AS, Bielack S, Doz F, McDermott R, Patel JD, Schilder RJ, Tahara M, Pfister SM, Witt O, Ladanyi M, Rudzinski ER, Nanda S, Childs BH, Laetsch TW, Hyman DM and Drilon A: Larotrectinib in patients with TRK fusion-positive solid tumours: a pooled analysis of three phase 1/2 clinical trials. Lancet Oncol *21(4)*: 531-540, 2020. PMID: 32105622. DOI: 10.1016/S1470-2045(19)30856-3

14 Drilon A, Nagasubramanian R, Blake JF, Ku N, Tuch BB, Ebata K, Smith S, Lauriault V, Kolakowski GR, Brandhuber BJ, Larsen PD, Bouhana KS, Winski SL, Hamor R, Wu WI, Parker A, Morales TH, Sullivan FX, DeWolf WE, Wollenberg LA, Gordon PR, Douglas-Lindsay DN, Scaltriti M, Benayed R, Raj S, Hanusch B, Schram AM, Jonsson P, Berger MF, Hechtman JF, Taylor BS, Andrews S, Rothenberg SM and Hyman DM: A next-generation TRK kinase inhibitor overcomes acquired resistance to prior TRK kinase inhibition in patients with TRK fusion-positive solid tumors. Cancer Discov *7(9)*: 963-972, 2017. PMID: 28578312. DOI: 10.1158/2159-8290.CD-17-0507

15 Gao Q, Chen CF, Dong Q, Hou L, Chen X, Zhi YL, Li X, Lu HT and Zhang HY: Elevated TrkA receptor expression is associated with all-trans retinoic acid-induced neuroblastoma differentiation. Genet Mol Res *14(4)*: 13195-13202, 2015. PMID: 26535632. DOI: 10.4238/2015.October.26.15

16 Shinwari Z, Al-Hindi H, Al-Shail E, Khafaga Y, Al-Kofide A, El-Kum N and Aboussekhra A: Response of medulloblastoma cells to vincristine and lomustine: role of TRKC, CTNNB1 and STK15. Anticancer Res *31(5)*: 1721-1733, 2011. PMID: 21617231.

17 Genevois AL, Ichim G, Coissieux MM, Lambert MP, Lavial F, Goldschneider D, Jarrosson-Wuilleme L, Lepinasse F, Gouysse G, Herceg Z, Scoazec JY, Tauszig-Delamasure S and Mehlen P: Dependence receptor TrkC is a putative colon cancer tumor suppressor. Proc Natl Acad Sci USA *110(8)*: 3017-3022, 2013. PMID: 23341610. DOI: 10.1073/pnas.1212333110

18 Kawamoto M, Onishi H, Ozono K, Yamasaki A, Imaizumi A, Kamakura S, Nakano K, Oda Y, Sumimoto H and Nakamura M: Tropomyosin-related kinase B mediated signaling contributes to the induction of malignant phenotype of gallbladder cancer. Oncotarget *8(22)*: 36211-36224, 2017. PMID: 28423707. DOI: 10.18632/oncotarget.16063

19 Kawamoto M, Ozono K, Oyama Y, Yamasaki A, Oda Y and Onishi H: The novel selective Pan-TRK inhibitor ONO-7579 exhibits antitumor efficacy against human gallbladder cancer *in vitro*. Anticancer Res *38(4)*: 1979-1986, 2018. PMID: 29599313. DOI: 10.21873/anticanres.12435

20 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(3)*: 205-214, 2013. PMID: 23312550. DOI: 10.1016/j.lungcan.2012.12.004

21 Odate S, Onishi H, Nakamura K, Kojima M, Uchiyama A, Kato M and Katano M: Tropomyosin-related kinase B inhibitor has potential for tumor regression and relapse prevention in pulmonary large cell neuroendocrine carcinoma. Anticancer Res *33(9)*: 3699-3703, 2013. PMID: 24023298.

22 Ozono K, Ohishi Y, Onishi H, Nakamura K, Motoshita J, Kato M, Nakanishi R, Nakamura M and Oda Y: Brain-derived neurotrophic factor/tropomyosin-related kinase B signaling pathway contributes to the aggressive behavior of lung squamous cell carcinoma. Lab Invest *97(11)*: 1332-1342, 2017. PMID: 28604655. DOI: 10.1038/labinvest.2017.45

23 Colucci-D'Amato L, Speranza L and Volpicelli F: Neurotrophic factor BDNF, physiological functions and therapeutic potential in depression, neurodegeneration and brain cancer. Int J Mol Sci *21(20)*: 7777, 2020. PMID: 33096634. DOI: 10.3390/ijms21207777

24 Ein L, Mei C, Bracho O, Bas E, Monje P, Weed D, Sargi Z, Thomas G and Dinh C: Modulation of BDNF-TRKB interactions on Schwann cell-induced oral squamous cell carcinoma dispersion *in vitro*. Anticancer Res *39(11)*: 5933-5942, 2019. PMID: 31704818. DOI: 10.21873/anticanres.13798

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