European Journal of Gynaecological Oncology

IMR Press

Original Research

Tropomyosin-related kinase B (TrkB) full-length isoform is related to advanced-stage clear cell ovarian cancer (CCOC)

Yumiko Goto[1,2,*], Yoshie Kametani[3,*], Aurélie Auguste[4], Thuraya Almamari[5], Audrey LeFormal[4], Shun-ichiro Izumi[1], Catherine Genestie[4,6], Hitoshi Ishimoto[1], Mikio Mikami[1], Alexandra Leary[4,5]

[1] *Department of Obstetrics and Gynecology, Tokai University School of Medicine, 259-1193 Kanagawa, Japan*

[2] *Department of Clinical Genetics, Tokai University Hospital, 259-1193 Kanagawa, Japan*

[3] *Department of Molecular Life Sciences, Tokai University School of Medicine, 259-1193 Kanagawa, Japan*

[4] *Gynecological Tumors Translational Research Lab, Gustave Roussy, 94805 Villejuif, France*

[5] *Department of Medical Oncology, Gustave Roussy, 94805 Villejuif, France*

[6] *Department of Biopathology, Gustave Roussy, 94805 Villejuif, France*

*Correspondence: ky49214@tsc.u-tokai.ac.jp (Yoshie Kametani); gotoyumi@is.icc.u-tokai.ac.jp (Yumiko Goto)

DOI:10.31083/j.ejgo4205137

This is an open access article under the CC BY 4.0 license (https://creativecommons.org/licenses/by/4.0/).

Submitted: 13 July 2021 Revised: 12 August 2021 Accepted: 18 August 2021 Published: 15 October 2021

Objective: Tropomyosin-related kinase B receptor (TrkB) is a receptor tyrosine kinase (RTK) that regulates the follicular growth and oocyte survival in the ovaries and is overexpressed in various cancers. Previously, we reported the increased expression of the TrkB isoform with tyrosine kinase (TrkB-TK) in Japanese clear cell ovarian cancer (CCOC) cases. In this study, we quantified TrkB isoform in French CCOC clinical samples and ovarian cancer cell lines to examine that the increased TrkB-TK expression was a common CCOC characteristics and to examine whether TrkB-TK associates with the resistance to cisplatin. *Methods*: The mRNA level of TrkB isoforms in twenty French CCOC cases and seventeen ovarian cancer cell lines involving twelve CCOC were quantified by real-time PCR. The expression of TrkB protein in twelve of these French CCOC samples were examined by immunohistochemistry (IHC). Four CCOC cell lines which expressed TrkB mRNA were selected, and *in vitro* cell viability assay was performed using a pan-Trk inhibitor K252a and cisplatin. *Results*: TrkB mRNA was expressed in all French CCOC cases and TrkB-TK was expressed at 70%. In advanced cases, TrkB-TK tended to increase. In addition, cell lines highly expressing TrkB-TK tended to be more cisplatin-sensitive under K252a treatment. *Discussion*: TrkB may be expressed in most of CCOC tissues. The TrkB-TK has a possibility to be involved in CCOC malignancy. Treatment with a pan-Trk inhibitor might be an adjuvant therapeutic strategy for patients with TrkB-TK expression including CCOC. *Conclusion*: TrkB was expressed in all French CCOC cases examined. High TrkB-TK expression was observed in advanced cases. TrkB-TK expressing CCOC cell lines tended to be more cisplatin-sensitive under K252a treatment.

Keywords

Tropomyosin-related kinase B (TrkB); Brain-derived neurotrophic factor (BDNF); Clear cell ovarian cancer (CCOC); Splicing variants; Cisplatin; Pan-Trk inhibitor

1. Introduction

Ovarian epithelial adenocarcinoma comprises four major pathological types: serous, endometrioid, mucinous and clear cell ovarian cancer (CCOC). Of these types, CCOC shows the highest resistance to chemotherapy and has a poor prognosis [1]. The increasing incidence rate of CCOC in Japan is concerning. Currently, this rate is higher than 25% among all ovarian cancers [2], which is higher than 4–12% in western countries [3]. The reason underlying its poor biological behavior has not been clarified yet.

Tropomyosin-related kinase B receptor (TrkB), a protein originally found in neural tissues, is expressed in normal decidua, villous tissue [4], endometrium and ovary [5–7]. TrkB is encoded by *NTRK2* gene and belongs to the Trk family which includes TrkA, TrkB and TrkC. The main ligand of TrkB is brain-derived neurotrophic factor (BDNF) [8]. Studies performed suggest that TrkB promotes cell growth and survival in the nervous system [9, 10]. Therefore, we hypothesized that the BDNF/TrkB signaling might play a role in the slowly developing, highly immortalized CCOC. In fact, overexpression of TrkB has been reported in ovarian cancer [11–13] and in various other cancer types such as neuroblastoma, pancreatic cancer, non-small cell lung cancer, hepatocellular carcinoma, colorectal cancer and so on [14–21]. Recently, *NTRK* fusions are the focus of research as a target of Entrectinib which is a potent inhibitor of TrkA, TrkB and TrkC [22].

TrkB has three major isoforms. TrkB-TK is a full-length isoform containing a tyrosine kinase (TK) domain in the intracellular region, and TrkB-T1 and TrkB-Shc are dominant negative isoforms lacking the TK domain [23, 24]. TrkB-TK participates in an intracellular signaling pathway activated by BDNF binding, and regulates cell differentiation, survival, and proliferation via RAS/MAPK and PI3K/AKT pathway [25–27]. K252a is a pan-Trk inhibitor which affects TrkB downstream signaling including PI3K/AKT and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). In

©2021 The Author(s). Published by IMR Press.

this study, we examined the sensitivity to cisplatin, standard anticancer drug for ovarian cancer, and to K252a in CCOC cell lines.

Truncated TrkB protein expression has been detected in the human endometrium [28, 29]. However, whether specific TrkB isoform expression in gynecological tissues contributes to gynecological cancer remains unclear. Therefore, we investigated the expression of different TrkB isoforms.

In our previous study with Japanese patients, we showed that all the ovarian carcinoma cases expressed TrkB-TK and observed the decreased expression of the truncated isoforms (TrkB-T1 and TrkB-Shc) in CCOC [29], which suggests that TrkB-TK expression may promote CCOC malignancy. Based on this, we aimed to elucidate whether the expression pattern of TrkB isoforms of European patients is the same as that of Japanese patients. CCOC incidence rate in France varies from 1% to 20% [30, 31]. The rate 20% seems higher than average rate of 4–12% in western countries, while the rate is similar to other European countries such as 21% in Italy [32]. In this study, we analyzed CCOC samples from French patients and several cancer cell lines to verify whether high expression of the TK domain is a common characteristic of CCOC in Japanese and French patients and if this contributed to the prognosis in CCOC.

2. Materials and methods

2.1 Ovarian carcinoma cases and cell lines

2.1.1 Clinical cases

Twenty CCOC tumor samples were examined using real-time PCR in this study. These samples were retrieved from surgical specimens obtained between 2011 and 2018 at Gustave Roussy Cancer Center (GRCC) (Villejuif, France). None of the patients had received preoperative chemotherapy. Participants were 50.5 $\pm$ 13.1 years of age. The histopathological diagnoses were carefully confirmed with a review by an expert pathologist in rare ovarian tumors. Clinical stage was determined according to the International Federation of Gynecology and Obstetrics Classifications (FIGO2014) and the TNM classification (Union for International Cancer Control). The survival data of patients were obtained from clinical records.

All patients signed a consent form, allowing use of their tissue for research. The project was approved by the Gustave Roussy Scientific Review Committee (CSET 2015-2290). Collected samples were processed and all experiments and analyses were conducted at the GRCC.

2.1.2 Cell lines

Five serous ovarian cancer cell lines (OVCAR3, OVCAR4, HUOA, SKOV, and COV), 12 CCOC cell lines (KOC, OVSAYO, OVISE, ES2, OVTOKO, TOV21G, OVMANA, RMG, SMOV, HUOCAII, KK, and HCH), other cancer cell lines including neuroblastoma (TGW), cervical cancer (HeLa), and human embryonic kidney (HEK293) cell line were used for qPCR analysis (Table 1). All of these cell lines are human origin. We obtained ES2 from American Type Culture Collection (ATCC) (Virginia, USA). TGW, HUOA, and HUOCAII were transferred from the Department of Obstetrics and Gynecology, Tokai University School of Medicine. The other cell lines were gifted from U981, INSERM.

Table 1. Cell lines.

Cell line	Origin of tissue	Character
OVCAR3	Ovary	Serous adenocarcinoma
OVCAR4		(n = 5)
HUOA		
SKOV3		
COV318		
KOC7C		Clear cell adenocarcinoma
OVSAYO		(n = 12)
OVISE		
ES2		
OVTOKO		
TOV21G		
OVMANA		
RMG		
SMOV		
HUOCAII		
KK		
HCH		
TGW	Adrenal gland	Neuroblastoma
HeLa	Cervix	Adenocarcinoma
HEK293	Embryonic kidney	-

2.2 Immunohistochemistry (IHC)

Whole-section tissue samples (4 μm thickness) were stained using anti-TrkB antibodies using a polymer peroxidase method as previously reported [29]. Briefly, after deparaffinization and rehydration, the sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After rinsing in PBS, the sections were incubated with the two anti-TrkB antibodies at 4 °C overnight. Mouse anti-human monoclonal antibody sc377218 (1:100; Santa Cruz Biotechnology, Texas, USA) was used to detect the extracellular region of all TrkB isoforms (TrkB-TK, TrkB-Shc, and TrkB-T1) and a rabbit anti-human polyclonal antibody ab18987 (1:50; Abcam, Cambridge, UK) to detect the tyrosine kinase domain specific to TrkB-TK (Fig. 1). An additional wash in PBS was followed by treatment with a peroxidase-labeled polymer conjugated to goat anti-mouse or anti-rabbit immunoglobulins (ENvision+kit; Dako, Agilent, California, USA) as the secondary antibody for 30 min at 24 °C. The signal was developed using diaminobenzidine, followed by counterstaining with hematoxylin. Tissue sections with primary antibodies replaced by PBS served as a negative control for each experiment. For each slide, the percentage of TrkB-positive tumor cells was evaluated by a pathologist (CG) at GRCC who specializes gynecological tumor.

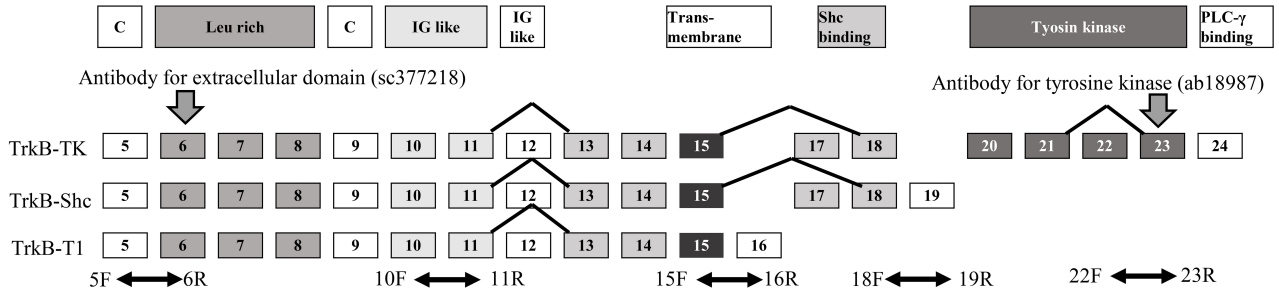

Fig. 1. Molecular structure of TrkB mRNA. Three major isoforms, TrkB-TK, TrkB-Shc and TrkB-T1, are shown. The box number represents the exon number. The primers used for detecting each domain are shown by black arrows. The names of the 10 primers and the domain fragments are also indicated. The 10 primers recognize 5 major domains of the TrkB molecule. Exon 5–6 is an extracellular domain close to the N terminus. Exon 10–11 contains a brain-derived neurotrophic factor (BDNF)-binding domain in the extracellular region. Exon 15–16 is an intracellular domain and the C terminus of the TrkB-T1 truncated isoform. Exon 18–19 contains a Shc-binding domain in the intracellular region and is the C terminal region specific for the TrkB-Shc truncated isoform. Exon 22–23 contains an intracellular tyrosine kinase domain specific for TrkB-TK. The two antibodies (sc377218 and ab18987) used for immunohistochemistry are indicated by gray arrows.

2.3 TrkB expression analysis by quantitative real-time PCR

2.3.1 Tissues

RNA was extracted from the core (2 μm thickness) of formalin-fixed, paraffin-embedded (FFPE) tumor samples using the RNeasy FFPE kit (QIAGEN, Venlo, Netherlands). Following cDNA synthesis using Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Fisher Scientific, Massachusetts, USA), RT-qPCR was conducted using Luminaris Color HiGreen Low ROX qPCR Master Mix (Thermo Fisher Scientific) and Viia7 Real-time PCR system (Applied Biosystems, Massachusetts, USA). The primers were designed to detect the main domains of TrkB to discriminate the three different TrkB isoforms. The housekeeping genes of HPRT1 were used as an endogenous control. All assays were performed in triplicates. Primers used in the study were as follows: Exon 5–6, 5′-TTCCGAGATTGGAGCCTAACAG-3′ and 5′-TCAGTCCCACATAAGCTTCAACA-3′;

Exon 10–11, 5′-CTGTAGTGTGGCAGGTGATCC-3′; 5′-TCTGCTTCCCACTGTCATCG -3′;

Exon 15–16, 5′-GTGGTGATTGCGTCTGTGGT-3′; 5′-CCCATCCAGTGGGATCTTATG-3′;

Exon 18–19, 5′-TCTTCGGAAGGTGGCCCAGAT-3′; 5′-GGGAACCTCTGGGCCATGT-3′;

Exon 22–23, 5′-GGGAGAACTTGCTGGTGAAA-3′; 5′-TTCCGTCGTGAATTTCCTGTA-3′;

HPRT1, 5′-TGCTTTCCTTGGTCAGGCAGTA-3′; 5′-TGGGGTCCTTTTCACCAGCAA-3′.

2.3.2 Cell lines

Cells were cultured in RPMI 1640 medium-GlutaMAXTM Supplement (Gibco, ThermoFisher Scientific, Massachusetts, USA) with 10% heat-inactivated fetal bovine serum (Gibco) and 5% penicillin-streptomycin (Gibco) in an atmosphere of 5% CO_2 at 37 °C. Twenty cell lines were analyzed by qPCR as previously described to determine TrkB isoform expression. Total RNA was extracted from approximately 3×10^6 cells using TRIzol Reagent (Invitrogen, ThermoFisher Scientific).

2.4 Treatment of CCOC cell lines with cisplatin and pan-Trk inhibitor K252a

CCOC cell lines, ES2, TOV21G, OVISE, and RMG were employed for *in vitro* assays. Cells were seeded on day0 into 96-well plates (Corning, Sigma-Aldrich, Missouri, USA) and cultured as described above. The cell concentration was 5000 cells/well for ES2 and TOV21G, 3000 cells/well for OVISE and RMG, and 4000 cells/well for HCH. Cells were treated with increasing doses of cisplatin (Bristol-Myers Squibb, NY, USA) (0.5, 5, and 10 μM) alone on day 1, which induces cancer cell apoptosis by combining with DNA and thereby inhibiting DNA duplication, or 100 nM K252a (Abcam)/well alone on day 1, or 100 nM K252a followed by cisplatin 4 h later, which inhibits PI3K/AKT pathway and CaMKII. CaMKII is activated by the Ca^{2+}/calmodulin complex and regulates the secretion of neurotrophin and transcription factors. All analyses were performed in triplicates. Cell numbers were counted on day 5 using CellTiter-Glo (Promega, Wisconsin, USA) and MikroWin2010 (Berthold Technologies, Bad WildBad, Germany) with CentroXS LB 960 (Berthold Technologies).

2.5 Statistics

Statistical analysis was performed using Excel 2016 software for Windows (Microsoft, Washington, USA). Differences in cell sensitivity to cisplatin in the presence or absence of K252a were analyzed by paired *t*-test. Differences were considered significant when the *p*-value was <0.05.

3. Results

3.1 Molecular structure of TrkB expressed by CCOC clinical cases

3.1.1 Molecular structure of TrkB mRNA expressed by CCOC clinical cases

Since molecular structure of TrkB is complicated, the detailed molecular structure of TrkB isoform could only be de-

Table 2. The expression of TrkB isoforms in French CCOC samples.

Clinical data					Real-time PCR (relative Ct/(SD))					IHC	
Samples	No.	Stage	Grade	Prognosis	ex5–6	ex10–11	ex15–16	ex18–19	ex22–23	ECR+ (%)	TKR+ (%)
CCOC (n = 20)	1	Ia	G3	ND	488.7 (0.707)	205.1 (0.857)	40.3 (0.432)	132.9 (1.603)	- (-)	ND	ND
	2	Ia	G3	ND	13.1 (0.412)	81.9 (0.323)	8.7 (0.187)	5.9 (1.344)	- (-)	60	0
	3	Ia	G3	ND	168.9 (0.266)	122.2 (0.789)	56.1 (0.297)	91.6 (0.747)	11.7 (1.152)	80	0
	4	Ic	G3	alive	10.7 (2.309)	29.1 (0.663)	5.6 (0.091)	19.6 (1.217)	3.0 (ND)	50	30
	5	Ic	G3	ND	91.1 (0.27)	98.1 (0.483)	46.0 (0.17)	46.1 (0.33)	- (-)	80	70
	6	Ic	G3	ND	458.8 (0.225)	230.1 (0.38)	653.0 (0.118)	23.4 (3.761)	14.0 (0.505)	60	20
	7	Ic	G3	dead	1366.1 (0.387)	305.7 (0.927)	125.0 (0.566)	66.7 (1.393)	31.5 (0.759)	ND	ND
	8	II	G3	dead	509.5 (0.133)	378.7 (0.171)	27.3 (2.836)	202.1 (0.298)	- (-)	40	60
	9	IIa	G3	alive	2291.1 (1.647)	299.2 (0.353)	86.9 (0.578)	154.9 (1.631)	- (-)	ND	ND
	10	IIc	G3	dead	1341.7 (0.724)	1074.1 (0.576)	136.5 (0.361)	626.4 (0.698)	134.0 (1.559)	ND	ND
	11	IIIc	G3	dead	111.7 (0.274)	130.5 (0.803)	54.6 (ND)	182.6 (0.115)	- (-)	60	0
	12	IIIc	G3	ND	105.5 (0.264)	157.6 (0.073)	56.9 (0.164)	9.7 (0.312)	1.0 (0.174)	70	70
	13	IIIc	G3	dead	298.6 (1.686)	95.3 (0.172)	18.1 (0.357)	27.0 (1.433)	4.7 (ND)	ND	ND
	14	IIIc	G3	dead	111.6 (0.505)	72.8 (0.573)	33.3 (0.852)	78.3 (0.635)	6.2 (ND)	70	60
	15	IIIc	G3	alive	181.1 (0.274)	186.6 (0.698)	152.6 (0.196)	22.0 (0.634)	7.4 (1.273)	80	50
	16	III	G3	dead	456.3 (1.318)	198.5 (0.822)	46.2 (1.487)	264.3 (0.979)	25.3 (ND)	ND	ND
	17	IV	G3	alive	6250.9 (2.105)	1553.0 (0.623)	135.6 (0.806)	326.0 (2.764)	160.3 (ND)	ND	ND
	18	ND	G3	alive	177.0 (0.906)	124.8 (1.659)	114.1 (1.218)	86.8 (1.195)	6.3 (ND)	20	0
	19	ND	G3	dead	148.2 (0.407)	207.3 (3.049)	39.3 (0.550)	67.2 (0.968)	6.5 (ND)	60	20
	20	ND	ND	ND	309.3 (0.361)	71.8 (1.244)	98.3 (0.316)	58.2 (1.467)	107.7 (ND)	ND	ND

Relative CT: The relative expression level (%) against HPRT1. The mRNA expression was quantified as $2^{-\delta Ct}$, where Ct = threshold cycle, δCt = (Ct target mRNA – Ct control HPRT1). ND, not determined; SD, standard deviation; ECR+ (%), percentage of extracellular positive cells; TKR+ (%), percentage of tyrosine kinase positive cells. Expression levels are classified by 0.0–9.9, 10.0–99.9, 100.0–999.9, 1000.0–, labeled from light to dark gray, respectively.

tected by RT-qPCR. Therefore, we examined the mRNA expression of TrkB in French CCOC cases using primers shown in Fig. 1. The clinical stage, grade, and prognosis are shown in Table 2.

As shown in Tables 2,3, 14/20 cases (70.0%) of the French CCOC cases expressed exon 22–23, which is involved in the TrkB-TK isoform. All French CCOC cases expressed exons 5–6, exon 10–11, exon 15–16 (a truncated TrkB-T1 isoform containing exon 16), and exon 18–19 (a truncated TrkB-Shc isoform with exon 19), suggesting that all samples expressed TrkB-T1 and TrkB-Shc. TrkB-TK isoform was found to be expressed in 5/10 cases (50.0%) of early stages I/II and in 6/7 cases (85.7%) of advanced stages III/IV (Table 2). Case No. 17 in stage IV expressed prominently higher TrkB-TK isoform mRNA compared to the other cases although it was not statistically significant (Table 2).

A. TrkB protein expression

Positive (%)	ECR+ (%)	TKR+ (%)
0<	0(0.0)	4(33.3)
10<	0(0.0)	0(0.0)
20<	1(8.3)	2(16.7)
30<	0(0.0)	1(8.3)
40<	1(8.3)	0(0.0)
50<	1(8.3)	1(8.3)
60<	4(33.3)	2(16.7)
70<	2(16.7)	2(16.7)
80<	3(25.0)	0(0.0)
90<	0(0.0)	0(0.0)
total	12(100.0)	12(100)

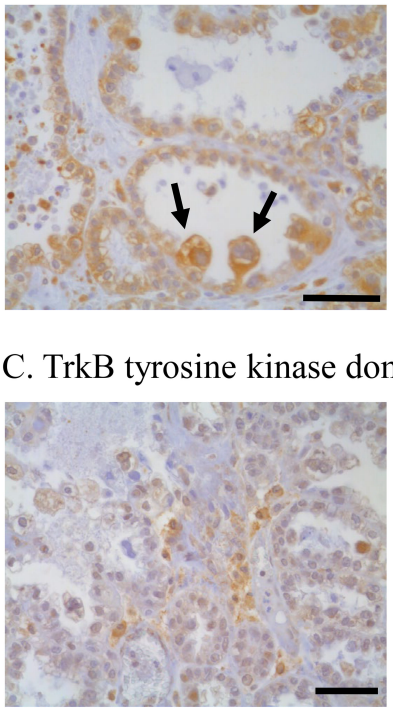

Fig. 2. Immunohistochemical analysis of TrkB in clear cell ovarian carcinoma (CCOC). TrkB protein expression by positive rate classification was shown (A). ECR+ means extracellular region positive cases and TKR+ means tyrosine kinase region positive cases. Representative CCOC case stained with anti-TrkB antibody specific for the extracellular (sc377218) (B) or tyrosine kinase (ab18987) (C) domain. Black arrows indicate the hobnail cells of CCOC tissues. Bars represent 10 μm. The case shown is No. 19 in Table 2.

Table 3. The percentage of TrkB expression by domain in French CCOC samples.

Samples	Relative CT	Exon 5–6 (%)	Exon 10–11 (%)	Exon 15–16 (%)	Exon 18–19 (%)	Exon 22–23 (%)
CCOC	-	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	6 (30.0)
(n = 20)	<0.01	0 (0.0)	0 (0.0)	2 (10.0)	2 (10.0)	7 (35.0)
	<0.10	3 (15.0)	6 (30.0)	12 (60.0)	11 (55.0)	4 (20.0)
	<1.0	13 (65.0)	12 (60.0)	6 (30.0)	7 (35.0)	3 (15.0)
	<10.0	4 (20.0)	2 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)
	<100.0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	total	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)

Collectively, all French CCOC cases expressed TrkB truncated isoforms TrkB-T1 and TrkB-Shc, and 70.0% of cases expressed TrkB-TK. The TrkB-TK expression ratio was high in cases with later stages in contrast to early stages.

3.1.2 TrkB protein expression in CCOC

As mRNA of TrkB contained TK domain in clinical samples, we confirmed the protein expression of TrkB in French CCOC samples by IHC. Among the 20 clinical samples, we examined the 12 FFPE samples. These samples were stained with anti-TrkB antibodies sc377218 and ab18987 (Fig. 1). As TrkB-T1 and TrkB-Shc do not contain the TK domain, we distinguished TrkB-TK and other TrkB isoforms by the expression of the extracellular domain and TK domain using these two antibodies.

The extracellular TrkB domain was detected in 100% of CCOC samples (n = 12/12), as shown in Fig. 2A, with 83.3% of the samples (n = 10/12) showing high levels of expression (>50% of tumor cells were positive). Analysis of TK domain expression revealed that 67.7% of CCOC samples (n = 8/12) were positive, with 41.7% of samples (n = 5/12) showing high expression levels (>50% of tumor cells were positive) (Fig. 2A). TrkB was localized in the cytoplasm in 91.7% for extracellular domain (n = 11/12 positive cases; sc377218) (Fig. 2B) and 63.6% for TK domain (n = 7/11 positive cases; ab18987), respectively (Fig. 2C). Moreover, Hobnail cells showed strong immunoreactivity to sc377218 (Fig. 2B).

Most of the IHC results corresponded to real-time PCR results. All the cases were positive for the extracellular region by IHC in parallel to exon 22–23 in real-time PCR. The ex-

pression of TK domain was almost the same between mRNA and protein in 66.7% of the cases (n = 8/12). Another two cases (No.3, No.18 in Table 2) were mRNA positive and protein negative for TK. The other two cases (No.5, No.8 in Table 2) were mRNA negative and protein positive for TK.

These results demonstrated that all the French CCOC cases expressed TrkB protein and TrkB-TK, although the TK domain was not expressed equally to the extracellular domain.

3.2 TK domain-dependence of cancer cell lines for survival against anticancer drugs

3.2.1 Molecular structure of TrkB mRNA in CCOC cell lines

To examine the effect of intracellular domains of TrkB *in vitro*, we selected ovarian cancer cell lines that expressed TrkB isoforms. First, we performed qPCR using ovarian cancer cell lines to analyze whether and which type of TrkB isoforms they expressed. A total of twenty cell lines, including CCOC, high-grade serous ovarian cancer (HGSOC) as a control of ovarian cancer other than CCOC, neuroblastoma (TGW) as a positive control of TrkB, and cervical cancer (HeLa) as a control of cancer other than ovarian cancer, were analyzed using the same protocol applied to CCOC clinical cases.

All cell lines expressed TrkB mRNA and all the CCOC cell lines (n = 12/12) expressed exon 22–23 (Table 4). Specifically, the expression of exon 22–23 was high in ES2 and RMG, intermediate in SMOV, OVTOKO, and HUOCAII, and low in TOV21G, OVMANA, and KK.

As shown in Table 4, most CCOC cell lines expressed higher level of exon 22–23 of TrkB-TK and exon 18–19 of TrkB-Shc than HGSOC cell lines. The levels of exon 15–16 of TrkB-T1 and exon 10–11 of BDNF-binding domain were almost the same in CCOC cell lines and HGSOC cell lines. CCOC cell lines expressed lower levels of TrkB extracellular domain exon 5–6 than HGSOC cell lines.

These results suggest that all the CCOC cell lines expressed intracellular domains, especially exon 22–23 and exon 18–19, which are important for signal transduction.

3.2.2 TK domain-dependence of cancer cell lines for survival against anticancer drugs

Next, we examined the sensitivity of the cell lines with high or low expression of the TK domain against the anticancer agents. The cell lines ES2, RMG, TOV21G and OVISE were selected as a representative cell line. ES2 and RMG highly expressed TrkB-TK mRNA which contains exon 22–23, and TOV21G and OVISE expressed TrkB-TK at low level (Table 4). The expression of TrkB-TK protein was observed in these four cell lines by western blotting using the antibody ab18987 (data not shown). As shown in Fig. 3, all four CCOC cell lines tested were significantly cisplatin sensitive. Significancy was $p = 0.00289$ (0.5 μM cisplatin), $p = 0.00006$ (5 μM cisplatin), $p = 0.00009$ (10 μM cisplatin) in ES2, $p = 0.00208$ (5 μM cisplatin), $p = 0.00196$ (10 μM cisplatin) in RMG, $p = 0.00221$ (0.5 μM cisplatin), $p = 0.00148$ (5 μM cisplatin), $p = 0.00149$ (10 μM cisplatin) in TOV21G, and $p = 0.00320$ (5 μM cisplatin), $p = 0.00278$ (10 μM cisplatin) in OVISE. Following single treatment with K252a, the viable cell number of ES2, RMG, and TOV21G cells were significantly decreased with $p = 0.00001$ (ES2, Fig. 3A), $p = 0.00809$ (RMG, Fig. 3B), and $p = 0.00143$ (TOV21G, Fig. 3C), suggesting that

Table 4. The mRNA expression of TrkB isoforms in ovarian cancer cell lines.

Cell lines		Real-time PCR (relative Ct/(SD))				
Type	Name	ex5–6	ex10–11	ex15–16	ex18–19	ex22–23
HGSOC	OVCAR3	465.2	1257.0	8908.7	834.0	906.1
(n = 5)		(0.053)	(0.203)	(0.17)	(0.74)	(1.088)
	OVCAR4	720.3	12.5	162.9	517.4	1709.4
		(0.156)	(0.222)	(ND)	(1.485)	(0.796)
	HUOA	0.5	1372.6	61333.6	2865.4	7236.2
		(0.233)	(0.151)	(1.200)	(0.736)	(0.823)
	SKOV3	424.4	78.7	276.4	329.4	3054.4
		(0.226)	(0.592)	(0.447)	(1.986)	(ND)
	COV318	839.1	1.0	-	20447.9	166.6
		(0.577)	(ND)	-	(ND)	(ND)
CCOC	KOC7C	0.0	17.4	92.8	38.0	2314.5
(n = 12)		(ND)	(0.911)	(0.040)	(2.240)	(0.674)
	OVSAYO	0.001	343.9	4103.6	3674.9	3206.2
		(0.043)	(0.183)	(0.724)	(0.763)	(0.268)
	OVISE	0.3	1572.0	15444.2	138365.3	2511.8
		(0.359)	(0.990)	(0.212)	(ND)	(0.137)
	ES2	447.7	4.7	129.2	67.2	202427.3
		(0.075)	(1.339)	(1.290)	(1.945)	(ND)
	OVTOKO	0.0	2.5	315.8	55518.9	7857.0
		(ND)	(ND)	(0.012)	(ND)	(0.414)
	TOV21G	0.000	18.6	205.5	2121.3	158.3
		(ND)	(0.896)	(ND)	(1.788)	(ND)
	OVMANA	0.000	18.0	493.1	436.4	43.2
		(0.839)	(0.685)	(ND)	(1.053)	(ND)
	RMG	0.015	2966.5	28081.2	896014.8	233394.8
		(0.996)	(0.700)	(0.224)	(0.973)	(1.750)
	SMOV	0.000	572.9	2279.5	15161.6	38121.4
		(ND)	(1.623)	(ND)	(0.442)	(0.865)
	HUOCAII	0.003	142.6	1057.1	260318.1	4668.8
		(0.564)	(ND)	(ND)	(1.205)	(1.311)
	KK	0.000	4.8	633.1	147.3	22.3
		(0.049)	(ND)	(2.390)	(0.387)	(0.072)
	HCH	0.000	2070.7	1036.7	2831781.9	4899.1
		(0.991)	(1.402)	(ND)	(ND)	(1.634)
Others	TGW	3.3	5289.8	108127.2	4534.4	58819.5
(n = 2)		(0.343)	(0.124)	(0.198)	(0.417)	(0.173)
	HeLa	194.0	33.0	212.5	222.1	17217.5
		(0.056)	(0.866)	(1.158)	(1.603)	(0.092)

Relative Ct: Relative expression level (%) against HEK293. The mRNA expression was quantified as $2^{-\delta\delta Ct}$, where Ct = threshold cycle, δCt = (Ct target mRNA – Ct control HPRT1), $\delta\delta$Ct = δCt target – δCt HEK293). ND, not determined; SD, standard deviation; ECR+ (%), percentage of extracellular positive cells; TKR+ (%), percentage of tyrosine kinase positive cells. Expression levels are classified by 1.0–9.9, 10.0–99.9, 100.0–999.9, 1000.0–9999.9, 10,000–, labeled from light to dark gray, respectively.

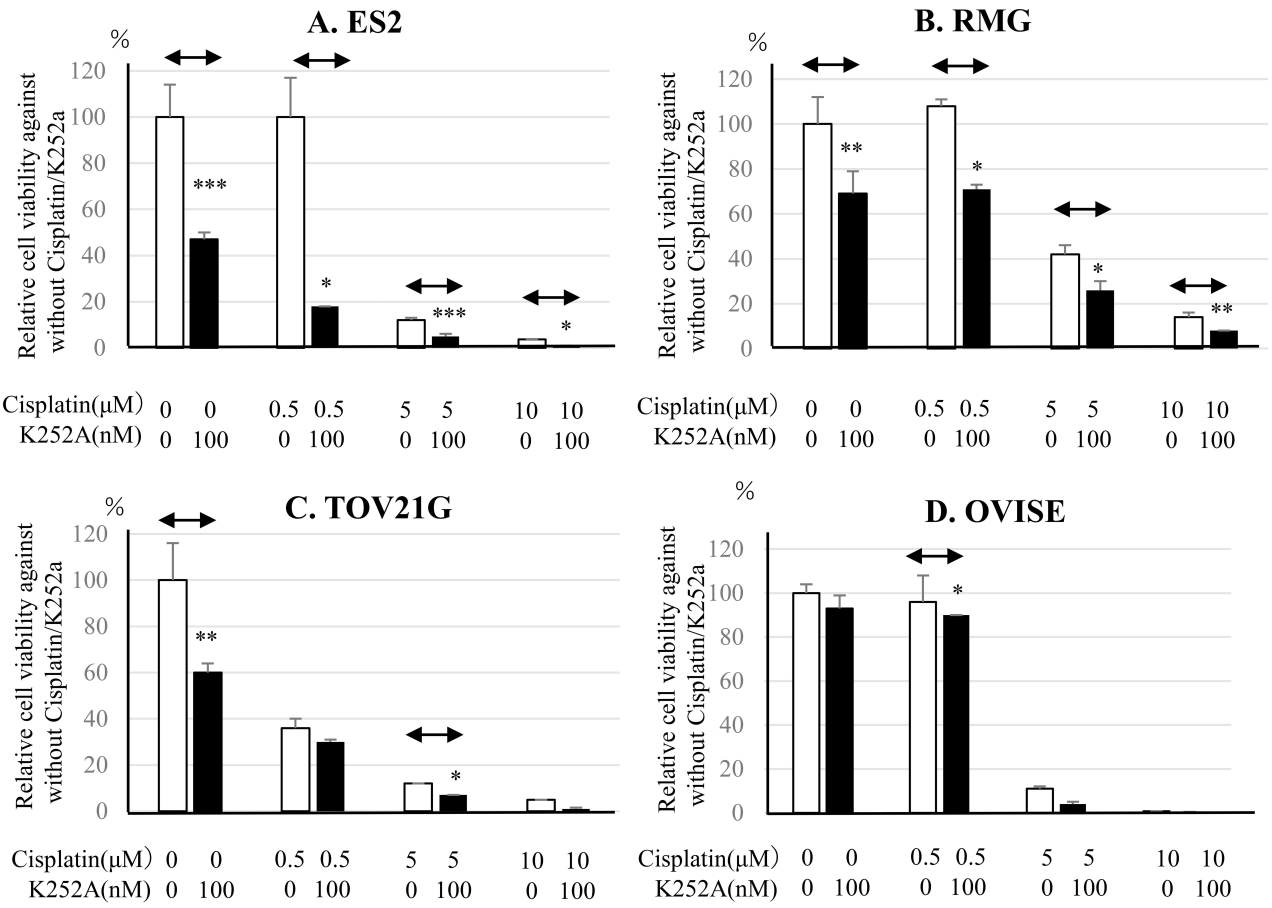

Fig. 3. Effect of K252a on the cisplatin sensitivity in clear cell ovarian carcinoma (CCOC) cell lines. The relative cell viability of ES2 (A), RMG (B), TOV21G (C), and OVISE (D) cells after Cisplatin/K252a treatment is shown. Each shows the relative viability (%) against the control culture. The control culture without Cisplatin/K252a is indicated as 100%. Open bars represent treatment without K252a. Closed bars show the treatment with K252a. Concentration of cisplatin (μM) and K252a (nM) are shown below in each histogram. Statistical significance is indicated as *($p < 0.05$), **($p < 0.01$), and ***($p < 0.001$).

TrkB affects cell survival and/or proliferation. Treatment with cisplatin significantly enhanced the sensitivity to K252a in ES2 (Fig. 3A) and RMG (Fig. 3B) cells at all concentrations of cisplatin, which expressed higher levels of TrkB-TK than TOV21G and OVISE (Table 4). In addition, the reduction in relative cell viability induced by K252a increased in a cisplatin concentration-dependent manner in ES2 (Fig. 3A) and RMG (Fig. 3B) cells. On the other hand, the cell viability reduction induced by K252a was not enhanced by increasing cisplatin concentrations in TOV21G (Fig. 3C) and OVISE (Fig. 3D) cells, which expressed TrkB-TK at low levels.

4. Discussion

In our previous study, we found that TrkB-TK was expressed in all Japanese CCOC samples. Here, we focused on the expression of TrkB isoforms in CCOC of French patients to examine their involvement in CCOC malignancy. Interestingly, we found that TrkB-TK was expressed at a relatively high rate in French patients with CCOC in advanced clinical stages. This suggests that the expression of TrkB isoforms, especially TrkB-TK, significantly affects CCOC malignancy.

In fact, Sclabas *et al.* [16] showed TrkB overexpression in metastatic pancreatic cancer cells. Au *et al.* [11] and Zheng *et al.* [12] reported the overexpression of TrkB in serous ovarian carcinoma. Moreover, Foster *et al.* [13] showed the overexpression of TrkB in endometriosis-associated ovarian cancer. However, not all studies examining TrkB expression in cancers confirmed its molecular structure. In our previous study, the molecular structure of the truncated TrkB isoform was frequently changed in CCOC [29].

The *TrkB* gene has an unusually large molecular structure of at least 590 kbp [23], contains 24 exons (Fig. 1), and tends to produce splicing variants. Stoilov *et al.* [23] demonstrated that the *TrkB* gene can generate at least 100 isoforms and can encode 10 proteins.

Abnormally processed RNA can induce carcinogenesis in various cancers [33]. Alternative splicing generates splice variants. Aberrant proteins can affect apoptosis, metastasis, and angiogenesis during carcinogenesis. Concerning TrkB splice variants, Pattwell *et al.* [34] showed that TrkB-T1

amplifies several oncogenic signaling pathways in human glioma. BDNF splice variants in human neuroblastoma have also been reported [35]. Nakagawara *et al.* [14] demonstrated the expression of TrkB-TK in immature neuroblastoma and of truncated TrkB in differentiated neuroblastoma. Nevertheless, the role of each TrkB splice variant in cancer remains unclear. Therefore, TrkB isoform analysis may be advantageous to clarify TrkB function.

In our previous study, first we showed the expression of TrkB in CCOC, and analyzed the expression of three different TrkB isoforms, TrkB-TK, TrkB-Shc, and TrkB-T1. The expression of TrkB-TK was observed in all Japanese CCOC cases, especially in advanced cases, while that of TrkB-Shc and TrkB-T1 was low [29].

Here, we observed expression of TrkB in all French CCOC cases and of TrkB-TK again in advanced cases. However, about 30% of the French CCOC cases did not express TrkB-TK protein or mRNA. On the other hand, the expression of TrkB-Shc and TrkB-T1 was observed in all French CCOC cases in contrast to Japanese cases, although the experimental method was not the same. In our previous study, we analyzed Japanese CCOC cases by semi-quantitative PCR not real-time PCR [29]. While it is difficult to compare the results, we used "real-time PCR" in this study because it provides high quantitativity. Considering this, TrkB-TK may not be necessary for CCOC oncogenesis, although this isoform could be an important factor in CCOC malignancy.

We need to discuss about the discrepancy of the result concerning TrkB-TK expression between mRNA and protein in several cases in this study (Table 2). It is possible that protein expression was not observed though the mRNA was expressed when the translation of TrkB mRNA was blocked. However, it may be difficult to explain the situation when the protein expression was observed though the mRNA was not expressed (No.5, No.8 in Table 2). As the reason, we have two hypotheses. One is the technical artifact. In this study, mRNA was extracted from FFPE samples. It might affect the yield and quality of mRNA obtained from FFPE samples, and the mRNA quality and quantity might not be sufficient for the real-time PCR. Otherwise, non-specific recognition by the antibody might have occurred. The other is the possibility of mRNA down-regulation after protein expression. After mRNA of tyrosine kinase had been down regulated, the CCOC cells might maintain the expression of TrkB protein with tyrosine kinase domain.

ES2 and RMG expressed relatively high mRNA levels of TK domain and could therefore be compared to advanced-stage clinical cases, which express relatively high TrkB-TK levels. With K252a treatment, the relative viability of ES2 and RMG cells was prominently decreased already at 0.5 μM cisplatin (Fig. 3). K252a might enhance cisplatin-induced cell apoptosis with an additive effect on TK domain-expressing cell lines because K252a and cisplatin have different mechanism to induce cell apoptosis. Though we need to examine how the inhibition of TrkB downstream cascade such as PI3K/AKT induced by K252a and the apoptosis induced by cisplatin affects the cell toxicity, the amount of cisplatin could be reduced in the treatment of advanced cases which express TrkB-TK. Decreased amount of cisplatin may reduce side effects and may improve the quality of life for patients.

The role of the truncated isoforms reported remains controversial. Truncated isoforms TrkB-Shc and TrkB-T1 inhibit TrkB-TK by trapping BDNF [36–38]. However, these truncated isoforms may not simply be dominant negative, but could also alter signal transduction [39]. In our results, ES2 were more sensitive to cisplatin, though both of ES2 and RMG highly expressed TrkB-TK; on the other hand, ES2 expressed low levels of TrkB-Shc with exon 18–19 (Table 4). In contrast, RMG were more cisplatin-resistant and expressed TrkB-Shc at high levels (Table 4). Therefore, the expression of TrkB-Shc might play a role in the survival of these cancer cells. Similarly, TOV21G (expressing low TrkB-TK and low TrkB-Shc levels) were more cisplatin-sensitive than OVISE (expressing low TrkB-TK and high TrkB-Shc levels) at 0.5 μM cisplatin (Fig. 3).

Although TrkB-Shc does not have a TK domain, high TrkB-Shc expression may be related to malignancy or cisplatin resistance because TrkB-Shc induces cell proliferation signaling by binding Shc [40].

5. Conclusions

We found that TrkB was highly expressed in French CCOC cases and observed TrkB-TK expression especially in advanced cases. In addition, cell lines expressing TrkB-TK tended to be more cisplatin-sensitive under pan-Trk inhibitor K252a treatment. Further analysis will be required to clarify the potential of K252a treatment as a targeted therapy for CCOC by inhibiting TrkB-TK signaling.

Author contributions

YG, AA and AL designed the case series and discussed the experimental method and the results. YG and ALF analyzed the data. TA collected the clinical information of the cases. CG examined all the result of IHC. SI, HI, and MM advised on the gynecological and oncological aspect. YG wrote the manuscript. AL and YK provided help and revised the corresponding sections in the manuscript. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Gustave Roussy Scientific Review Committee (approval number: CSET 2015-2290).

Acknowledgment

We would like to express our gratitude to all those who helped us during the writing of this manuscript especially to

the members of INSERM981 unit and the support center for medical research and education of Tokai University. Thanks to all the peer reviewers for their opinions and suggestions.

Funding

This project was supported by the Canon Foundation in Europe Fellowship for 2017 (YG) and the MEXT KAKENHI (Grant No.: JP17K11298) for 2017-2019 (YG).

Conflict of interest

The authors declare no conflict of interest.

References

[1] Sugiyama T, Kamura T, Kigawa J, Terakawa N, Kikuchi Y, Kita T, *et al.* Clinical characteristics of clear cell carcinoma of the ovary. Cancer. 2000; 88: 2584–2589.

[2] Fujiwara K, Shintani D, Nishikawa T. Clear-cell carcinoma of the ovary. Annals of Oncology. 2016; 27: i50–i52.

[3] Itamochi H, Kigawa J, Terakawa N. Mechanisms of chemoresistance and poor prognosis in ovarian clear cell carcinoma. Cancer Science. 2008; 99: 653–658.

[4] Fujita K, Tatsumi K, Kondoh E, Chigusa Y, Mogami H, Fujii T, *et al.* Differential expression and the anti-apoptotic effect of human placental neurotrophins and their receptors. Placenta. 2011; 32: 737–744.

[5] Paredes A, Romero C, Dissen GA, DeChiara TM, Reichardt L, Cornea A, *et al.* TrkB receptors are required for follicular growth and oocyte survival in the mammalian ovary. Developmental Biology. 2004; 267: 430–449.

[6] Kawamura K, Kawamura N, Mulders SM, Sollewijn Gelpke MD, Hsueh AJW. Ovarian brain-derived neurotrophic factor (BDNF) promotes the development of oocytes into preimplantation embryos. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102: 9206–9211.

[7] Anderson RA, Bayne RAL, Gardner J, De Sousa PA. Brain-derived neurotrophic factor is a regulator of human oocyte maturation and early embryo development. Fertility and Sterility. 2010; 93: 1394–1406.

[8] Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziejewski C, *et al.* TrkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell. 1991; 65: 885–893.

[9] Klein R, Conway D, Parada LF, Barbacid M. The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell. 1990; 61: 647–656.

[10] Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, *et al.* The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. Cell. 1991; 65: 895–903.

[11] Au CWH, Siu MKY, Liao X, Wong ESY, Ngan HYS, Tam KF, *et al.* Tyrosine kinase B receptor and BDNF expression in ovarian cancers – Effect on cell migration, angiogenesis and clinical outcome. Cancer Letters. 2009; 281: 151–161.

[12] Zheng W, Dai Q, Tao P, Sun A, Wang Y, Bao L, *et al.* Overexpression of tyrosine kinase receptor B promotes metastasis of ovarian serous adenocarcinoma by lymphangiogenesis. Tumori. 2011; 97: 756–761.

[13] Foster WG, Elias R, Faghih M, Dominguez MA, Elit L, Boutross-Tadross O. Immunohistochemical localization of tyrosine receptor kinases a and B in endometriosis-associated ovarian cancer. Histopathology. 2009; 54: 907–912.

[14] Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM. Expression and function of TRK-B and BDNF in human neuroblastomas. Molecular and Cellular Biology. 1994; 14: 759–767.

[15] Thiele CJ, Zhijie Li, McKee AE. On Trk—the TrkB signal transduction pathway is an increasingly important target in cancer biology. Clinical Cancer Research. 2009; 15: 5962–5967.

[16] Sclabas GM, Fujioka S, Schmidt C, Li Z, Frederick WA, Yang W, *et al.* Overexpression of tropomysin-related kinase B in metastatic human pancreatic cancer cells. Clinical Cancer Research. 2005; 11: 440–449.

[17] Miknyoczki SJ, Lang D, Huang L, Klein-Szanto AJ, Dionne CA, Ruggeri BA. Neurotrophins and Trk receptors in human pancreatic ductal adenocarcinoma: expression patterns and effects on *in vitro* invasive behavior. International Journal of Cancer. 1999; 81: 417–427.

[18] Harada T, Yatabe Y, Takeshita M, Koga T, Yano T, Wang Y, *et al.* Role and Relevance of TrkB Mutations and Expression in Non–Small Cell Lung Cancer. Clinical Cancer Research. 2011; 17: 2638–2645.

[19] Pearse RN, Swendeman SL, Li Y, Rafii D, Hempstead BL. A neurotrophin axis in myeloma: TrkB and BDNF promote tumor-cell survival. Blood. 2005; 105: 4429–4436.

[20] Lam C, Yang Z, Lau C, Tam K, Fan S, Poon RTP. Brain-derived neurotrophic factor promotes tumorigenesis via induction of neovascularization: implication in hepatocellular carcinoma. Clinical Cancer Research. 2011; 17: 3123–3133.

[21] Fujikawa H, Tanaka K, Toiyama Y, Saigusa S, Inoue Y, Uchida K, *et al.* High TrkB expression levels are associated with poor prognosis and EMT induction in colorectal cancer cells. Journal of Gastroenterology. 2012; 47: 775–784.

[22] Doebele RC, Drilon A, Paz-Ares L, Siena S, Shaw AT, Farago AF, *et al.* Entrectinib in patients with advanced or metastatic NTRK fusion-positive solid tumours: integrated analysis of three phase 1–2 trials. The Lancet Oncology. 2020; 21: 271–282.

[23] Stoilov P, Castren E, Stamm S. Analysis of the human TrkB gene genomic organization reveals novel TrkB isoforms, unusual gene length, and splicing mechanism. Biochemical and Biophysical Research Communications. 2002; 290: 1054–1065.

[24] Luberg K, Wong J, Weickert CS, Timmusk T. Human TrkB gene: novel alternative transcripts, protein isoforms and expression pattern in the prefrontal cerebral cortex during postnatal development. Journal of Neurochemistry. 2010; 113: 952–964.

[25] Barnabé-Heider F, Miller FD. Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. The Journal of Neuroscience. 2003; 23: 5149–5160.

[26] Nonomura T, Kubo T, Oka T, Shimoke K, Yamada M, Enokido Y, *et al.* Signaling pathways and survival effects of BDNF and NT-3 on cultured cerebellar granule cells. Developmental Brain Research. 1996; 97: 42–50.

[27] McCarty JH, Feinstein SC. The TrkB receptor tyrosine kinase regulates cellular proliferation via signal transduction pathways involving SHC, PLCgamma, and CBL. Journal of Receptor and Signal Transduction Research. 1999; 19: 953–974.

[28] Wessels JM, Wu L, Leyland NA, Wang H, Foster WG. The brain-uterus connection: brain derived neurotrophic factor (BDNF) and its receptor (Ntrk2) are conserved in the mammalian uterus. PLoS ONE. 2014; 9: e94036.

[29] Goto Y, Kametani Y, Kikugawa A, Tsuda B, Miyazawa M, Kajiwara H, *et al.* Defect of tropomyosin-related kinase B isotype expression in ovarian clear cell adenocarcinoma. BioScience Trends. 2014; 8: 93–100.

[30] Mackay HJ, Brady MF, Oza AM, Reuss A, Pujade-Lauraine E, Swart AM, *et al.* Prognostic relevance of uncommon ovarian histology in women with stage III/IV epithelial ovarian cancer. International Journal of Gynecological Cancer. 2010; 20: 945–952.

[31] Delvallée J, Cancel M, Body G, Bendifallah S, Touboul C, Dabi Y, *et al.* Lymphovascular invasion as a criterion for adjuvant chemotherapy for FIGO stage i-IIa clear cell carcinoma, mucinous, low grade serous and low grade endometrioid ovarian cancer. Journal of Gynecology Obstetrics and Human Reproduction. 2021; 50: 102193.

[32] Calura E, Ciciani M, Sambugaro A, Paracchini L, Benvenuto G, Milite S, *et al.* Transcriptional Characterization of Stage I Epithelial Ovarian Cancer: A Multicentric Study. Cells. 2019; 8: 1554.
[33] Sveen A, Kilpinen S, Ruusulehto A, Lothe RA, Skotheim RI. Aberrant RNA splicing in cancer; expression changes and driver mutations of splicing factor genes. Oncogene. 2016; 35: 2413–2427.
[34] Pattwell SS, Arora S, Cimino PJ, Ozawa T, Szulzewsky F, Hoellerbauer P, *et al.* A kinase-deficient NTRK2 splice variant predominates in glioma and amplifies several oncogenic signaling pathways. Nature Communications. 2020; 11: 2977.
[35] Aoyama M, Asai K, Shishikura T, Kawamoto T, Miyachi T, Yokoi T, *et al.* Human neuroblastomas with unfavorable biologies express high levels of brain-derived neurotrophic factor mRNA and a variety of its variants. Cancer Letters. 2001; 164: 51–60.
[36] Wong J, Garner B. Evidence that truncated TrkB isoform, TrkB-Shc can regulate phosphorylated TrkB protein levels. Biochemical and Biophysical Research Communications. 2012; 420: 331–335.
[37] Haapasalo A, Koponen E, Hoppe E, Wong G, Castrén E. Truncated trkB.T1 is dominant negative inhibitor of trkB.TK+-mediated cell survival. Biochemical and Biophysical Research Communications. 2001; 280: 1352–1358.
[38] Eide FF, Vining ER, Eide BL, Zang K, Wang XY, Reichardt LF. Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. The Journal of Neuroscience. 1996; 16: 3123–3129.
[39] Fenner BM. Truncated TrkB: beyond a dominant negative receptor. Cytokine & Growth Factor Reviews. 2012; 23: 15–24.
[40] Baxter GT, Radeke MJ, Kuo RC, Makrides V, Hinkle B, Hoang R, *et al.* Signal Transduction Mediated by the Truncated trkB Receptor Isoforms, trkB.T1 and trkB.T2. The Journal of Neuroscience. 1997; 17: 2683–2690.