Original Article

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Defect of tropomyosin-related kinase B isotype expression in ovarian clear cell adenocarcinoma

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Summary **Tropomyosin-related kinase B (TrkB) is a functional signal molecule that correlates with cell survival and epithelial-mesenchymal transition (EMT), which is essential for the invasiveness of malignant cancer cells. While a truncated isoform of TrkB has a dominant negative effect, full-length TrkB with its tyrosine kinase domain is predicted to play a role in cancer progression. Because ovarian clear cell adenocarcinoma (CCA) shows worse prognosis compared to other cancer types, we investigated the correlation between TrkB isoforms and the progression of CCA. Ovarian adenocarcinoma and benign tumor samples were obtained from Tokai University Hospital and Juntendo University Hospital. These samples were examined for the TrkB expression of isotype-specific proteins and mRNAs by immunohistochemistry and domain-specific semi-quantitative reverse transcription polymerase chain reaction. While TrkB mRNA expression was detected in all of the ovarian tissues and TrkB protein expression was predominant in ovarian cancer tissues, the number of tissues expressing the tyrosine kinase-truncated isoforms (T-Shc or T1) decreased according to the clinical stage of CCA. Irregular isoforms were also observed in some CCA samples. The decrease in T-Shc and T1 were less obvious in mucinous adenocarcinoma and not observed in serous or endometrioid adenocarcinoma. Decreased expression of the truncated isoforms (T-Shc and T1) was associated with CCA progression. These results demonstrate that irregular expression of TrkB isoforms is a characteristic of CCA tissues. The unique TrkB expression profile may be useful for the diagnosis of CCA subtypes.**

Keywords: Tropomyosin-related kinase B, brain-derived neurotrophin factor, ovarian cancer, clear cell adenocarcinoma, isoform

1. Introduction

Brain-derived neurotrophin factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB) belong to the nerve growth factor family and the Trk family, respectively (*1,2*). TrkB was first reported to be a BDNF receptor and to support the growth and maintenance of the nervous system. Thereafter, TrkB and BDNF were found to be expressed in gynecological tissues including the ovary, particularly in oocytes (*3,4*). In the normal ovary, BDNF/TrkB signaling plays a supportive role in the growth of follicles (*3*) and fertilized eggs (*4*) through BDNF secreted from granulosa cells and cumulus cells, as it stimulates TrkB receptors expressed on oocytes. TrkB signaling reportedly promotes cell survival and epithelial-mesenchymal transition (EMT) (*5,6*) in head and neck squamous cell carcinoma. These features of

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TrkB activity and its primary role as a growth factor receptor are presumably related to carcinogenesis (*7,8*). In fact, overexpression of TrkB has been observed in various malignant tumors, including neuroblastoma, prostate cancer, pancreatic cancers, multiple myeloma and pulmonary carcinoid tumors.

The *TrkB* gene (Figure 1) is relatively large, spanning more than 350 kbp and containing 24 exons (*9*). Full-length TrkB has an intracellular tyrosine kinase (TK) domain. In human neural tissue, two C-terminal truncated TrkB receptors, TrkB-T-Shc and TrkB-T1, are also expressed (*10,11*), and both act as dominant-negative inhibitors of the full-length receptor (*12*). These truncated isoforms, TrkB-T-Shc and TrkB-T1, may function in normal neuronal tissues to modulate the TrkB signal. Moreover, TrkB isoforms lacking several exons at the N-terminus have recently been predicted by in silico analysis (*13*).

Ovarian carcinoma is usually classified into four histopathological types: serous, endometrioid, mucinous, and clear cell adenocarcinoma (CCA). There have been many reports comparing the features of these cancer types (*14*). Among these ovarian cancer types, CCA accounts for more than 20% of ovarian carcinomas in Japan, where its frequency is higher compared to western countries (*15*). CCA has a worse prognosis compared to other types of ovarian carcinoma, as CCA is resistant to various anti-cancer treatments, although its growth is slow. These characteristics are similar to those of cancer stem cells, which might be induced by the process of EMT (*16*). However, the molecular mechanisms of the worse biological behavior of CCA have not been fully clarified.

Large molecules with multiple functional domains such as TrkB can have several variants as a result of splicing or mutation of the DNA caused by oncogenesis. To date, the over-expression of TrkB protein in cancers has been evaluated by immunohistochemical analysis (*17*). However, any major abnormalities of the molecular structure of TrkB have not been reported in cancer tissues.

In the present study, ovarian cancer tissues were first examined for structural abnormalities in TrkB mRNA to determine whether a decrease in dominant-negative isoforms occurs with CCA progression. The results indicate that the expression of irregular TrkB variants could be a potential marker of CCA diagnosis and a target of CCA treatment.

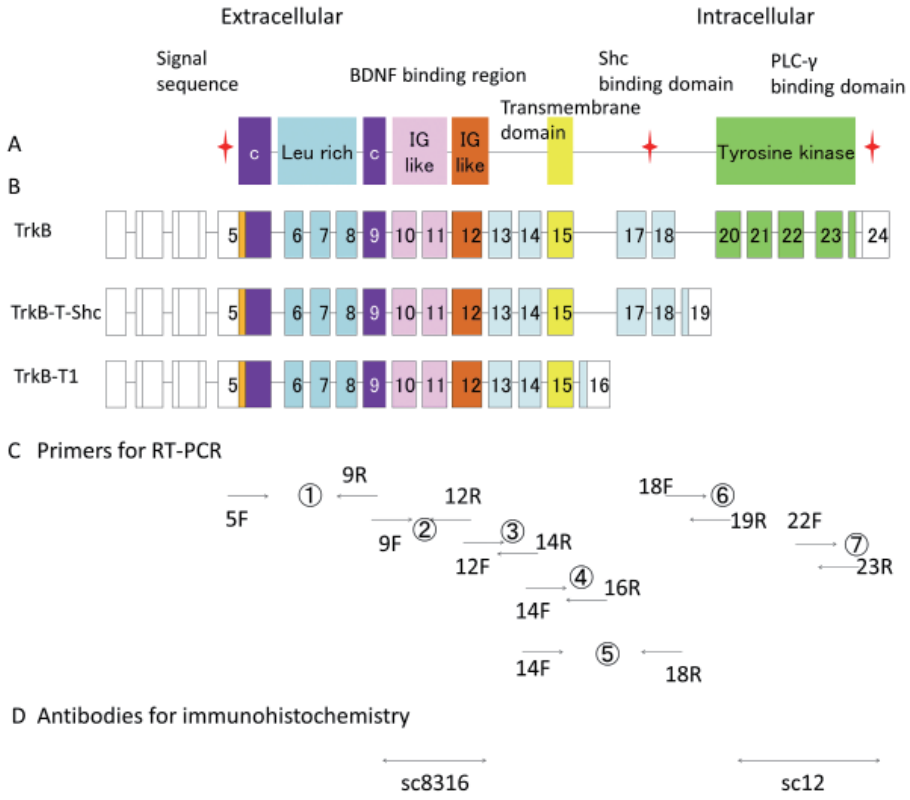

Figure 1. Predicted TrkB protein isoforms with primers and antibodies used for analysis. (A) Domains of full-length TrkB protein. c, cysteine-rich region; Leu rich, leucine-rich region; IG like, immunoglobulin-like domain. **(B)** Major splice variants of TrkB. TrkB: full-length TrkB; TrkB-T-Shc: TrkB with the Shc binding domain but without the kinase and PLC-γ domains; TrkB-T1: TrkB without functional intracellular domains. The 5' and 3' untranslated region domains are shown as empty boxes. The coding region is shown as boxes that are colored differently for each domain. **(C)** The locations of the primers for RT-PCR are shown by arrows, and the numbers indicate the amplified fragments. Thus, each primer is labeled with the exon number, and the forward (F) or reverse (R) direction is also indicated by the direction of the arrow. **(D)** Epitopes of the antibodies (sc8316 and sc12) used for immunohistochemical analysis are indicated by double-headed arrows.

2. Materials and Methods

2.1. *Clinical samples*

Immunohistochemical specimens of ovarian cancer (n = 105), endometriosis (n = 17), benign ovarian tumors (n = 9) and normal endometrium (n = 28) were obtained from patients who underwent surgery at Tokai University Hospital (Kanagawa, Japan) from 1994 to 2010 (Table 1A). RNA was extracted from fresh-frozen specimens of ovarian cancer tissue following reverse transcription polymerase chain reaction (RT-PCR) (n = 47) (Table 1B). All specimens contained at least 70% tumor tissue. Tumors were harvested peri-operatively from 2007 to 2011 at Tokai University Hospital and Juntendo University Hospital (Tokyo, Japan) and immediately frozen at -80°C. All tissues were obtained with the informed consent of the patients, and this study was approved by the Institutional Review Boards of Tokai University and Juntendo University.

2.2. *Cell culture*

Ovarian cancer cell lines and a neuroblastoma cell line (TGW) were purchased from American Type Culture Collection (Manassas, VA, USA). TGW was cultured at 37°C under 5% CO_2 in Glutamax with 10% fetal bovine serum (Equitech-Bio, Inc., Kerrville, TX, USA), and 10 mM all trans retinoic acid (Sigma-Aldrich, St. Louis, MO, USA) was added to induce TrkB expression. Cells were grown in 10 mm dishes (Asahi Glass Co., Ltd., Tokyo, Japan) coated with type I collagen for 6 days, after which RNA was extracted to use as a positive control for RT-PCR.

2.3. *Immunohistochemistry*

Sections of formalin-fixed, paraffin-embedded tissues were stained using 2 polyclonal antibodies for TrkB (Figure 1): anti-TrkB antibody sc8316, which targets the extracellular domain near the BDNF-binding site (N: 160-340, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-TrkB antibody sc12, which targets the TK domain (Santa Cruz). One monoclonal antibody was used to stain for BDNF (anti-BDNF mouse antibody, clone #35928.11; Merck, Darmstadt, Germany). Biotinylated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) (1:300) was used as the secondary antibody for sc8316, and biotinylated anti-mouse IgG (Fitzgerald, Acton, MA, USA) (1:200) was used for BDNF. Sections were stained by the ABC method (Vector Laboratories Inc., Burlingame, CA, USA) for the sc8316 and anti-BDNF antibodies and by the EnVision method (Dako, Glostrup, Denmark) for the sc12 antibody. EnVision™+ Rabbit/HRP (Dako) was used for 1 hour for sc12.

Sections of ovarian CCA specimens with strong staining for sc8316 and sc12 and sections of cerebellar tissue specimens that strongly stained for BDNF were used as positive controls. For the negative control, we used rabbit IgG (sc8316 and sc12) or mouse IgG (BDNF) in place of the primary antibodies. If more than 10% of the tumor cells were positive for TrkB or BDNF, the tumor was classified as positive.

Table 1. Clinical characteristics of the patients

A. Clinical characteristics of the patients. IHC analysis

Items	Number	Age (mean ± S.D.)	Stage			
			I	II	III	IV/rec
Ovarian cancer	105	54.6 ± 10.0	55	10	32	8
Mucinous	11	53.7 ± 16.4	7	2	2	0
Serous	19	58.7 ± 8.7	5	0	11	3
Endometrioid	25	53.1 ± 11.3	13	3	6	3
Clear cell	50	54.0 ± 7.7	30	5	13	2
Benign tissue	51	46.4 ± 8.6				
Endometriosis	17	43.8 ± 7.0				
Benign ovarian tumors	9	54.8 ± 13.8				
Endometrium	28	45.2 ± 5.6				

(OA: n = 105, EM: n = 17, BOT: n = 9, NE: n = 28)

B. Clinical characteristics of the patients. RT-PCR analysis

Items	Number	Age (mean ± S.D.)	Stage			
			I	II	III	IV/rec
Ovarian cancer	47	53.6 ± 11.8	22	6	16	4
Mucinous	8	50.8 ± 18.5	7	1	0	0
Serous	8	62.0 ± 12.4	1	0	7	0
Endometrioid	10	54.5 ± 9.0	2	3	5	1
Clear cell	21	51.1 ± 8.6	12	2	4	3

(OA: n = 47,)

2.4. *RT-PCR and DNA sequence analysis*

Total RNA was extracted using TRIzol LS reagent (Invitrogen, San Diego, CA, USA) or a Qiagen extraction kit (Qiagen, Valencia, CA, USA) from fresh-frozen tissues or cultured cells according to the manufacturer's instructions. Then, the optical density of the extracts was measured, and the concentration to use for RT-PCR was selected. The primers for TrkB (Figure 1), BDNF, and β-actin are as indicated in the Supplemental data (*http://www.biosciencetrends.com/docindex.php?year=2014&kanno=2*). Amplification involved initial reverse transcription at 50°C for 30 min and 95°C for 15 min, followed by 33 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C. Final extension was then carried out for 5 min at 72°C, after which PCR products were separated by electrophoresis on a 1% agarose gel in 1 × Tris-Borate-EDTA (TBE) and stained with 0.5 μg/mL ethidium bromide.

Direct DNA sequencing of the PCR products was performed using the primers above and an Applied Biosystems 3500XL sequencer (Applied Biosystems, Carlsbad, CA, USA). Each sequence was compared with those registered in the National Center for Biotechnology Information (NCBI) database, and A Plasmid Editor (ApE) software was used for this analysis.

2.5. *Statistical analysis*

Statistical analyses were conducted using Microsoft Office Excel 2007 (Microsoft, Redmond, WA, USA). Differences were evaluated by the chi-square test for comparison among tumor subtypes or stages. The results were considered to be significant if the *p*-value was < 0.05.

3. Results

We first used the extracellular domain-specific antibody (Ab) sc8316 and the intracellular domain-specific Ab sc12 to examine the localization of TrkB proteins in the tissues by immunohistochemistry. As shown in Figures 2A and 2B, the cell membrane and cytoplasm of ovarian CCA cells were clearly stained by sc8316. The invasive front of the tumor showed greater expression of TrkB (Figure 2B). As with sc8316, the cell membrane and cytoplasm were both stained by sc12 antibody (Figure 2C). These results suggest that TrkB expression was increased on CCA cells in invasive sites.

As shown in Table 2, the percentage of TrkB-positive ovarian cancers was 74.3%, which was significantly higher when compared with endometriotic tissue and normal endometrium ($p < 0.05$). Immunohistochemical analysis did not indicate a significant tissue type or clinical stage specificity. We further examined the protein expression of the intracellular domain using

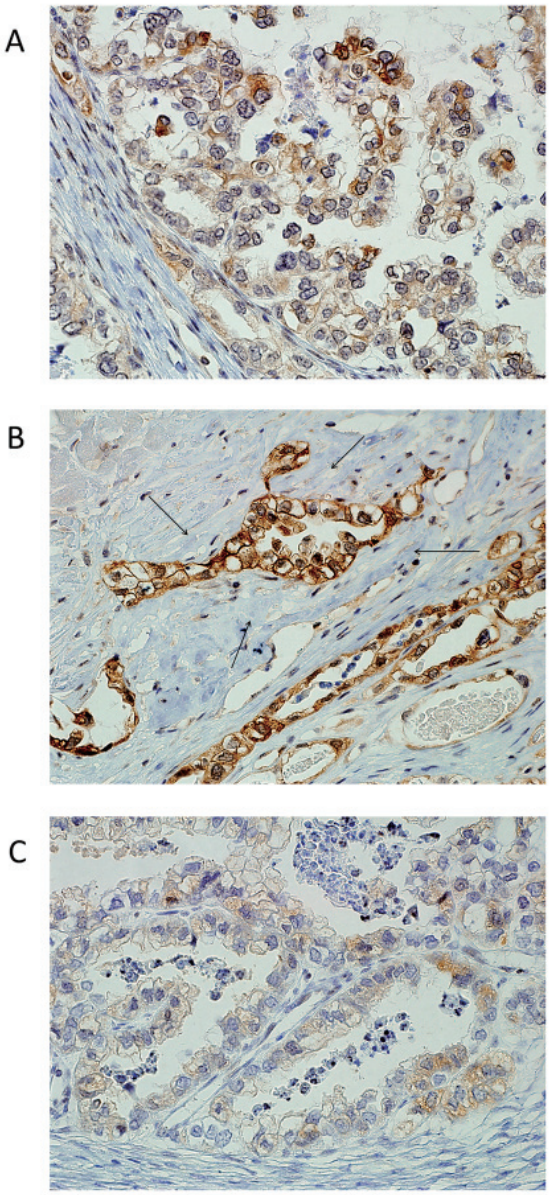

Figure 2. Immunohistochemical analysis of TrkB in ovarian cancer. (A) Representative photomicrograph of a primary clear cell adenocarcinoma stained with sc8316 (patient number 30 in Figure 4). (×20). **(B)** Representative photomicrograph of the invasive front of a clear cell adenocarcinoma stained with sc8316 (patient number 30 in Figure 4). Arrows indicate sites of strong TrkB expression at the invasive front. (×20). **(C)** Representative photomicrograph of clear cell adenocarcinoma stained with sc12 (patient number 30 in Figure 4). (×20). An Olympus AX70 optical microscope, DP2-BSW software (Ver 2.1), and DP72 camera were used.

sc12. Similar to sc8316, the cell surface and cytoplasm were stained positively by the antibody (Figure 1D). Among the 78 TrkB-positive cases, the percentage of sc8316 and sc12 double-positive cancers was 19.2% (15 cases). TK domain expression was observed in ovarian cancers and benign tumors but was not observed in the endometrium or endometriotic tissue. The percentage of sc12-positive cases did not differ among tissue types or clinical stages (Table 2).

The percentage of BDNF-positive ovarian cancers

Table 2. Immunohistochemical analysis of TrkB isoform and BDNF expression in clinical samples

Items	Expression of TrkB (sc8316)			Expression of TrkB (sc12)			Expression of BDNF		
	Negative (-)	Positive (+)	Total	Negative (-)	Positive (+)	Total	Negative (-)	Positive (+)	Total
Benign tissues									
Endometriosis	9 (52.9%)	8 (46.1%)	17	8 (100%)	0 (0%)	8	2 (11.8%)	15 (88.2%)	17
Endometrium	15 (53.6%)	13 (46.4%)	28	13 (100%)	0 (0%)	13	12 (42.8%)	16 (57.2%)	28
Ovarian tumor	1 (11.1%)	8 (88.9%)	9	6 (66.7%)	3 (33.3%)	9	1 (11.1%)	8 (88.9%)	9
Ovarian cancer									
Total	27 (25.7%)	78 (74.3%)	105	63 (80.8%)	15 (19.2%)	78	17 (16.2%)	88 (83.8%)	105
Mucinous	1 (9.1%)	10 (90.9%)	11	7 (70.0%)	3 (30.0%)	10	1 (9.1%)	10 (90.9%)	11
Serous	4 (21.0%)	15 (79.0%)	19	12 (80.0%)	3 (20.0%)	15	1 (5.3%)	18 (94.7%)	19
Endometrioid	13 (52.0%)	12 (48.0%)	25	11 (91.7%)	1 (8.3%)	12	11 (44.0%)	14 (56.0%)	25
Clear cell	9 (18.0%)	41 (82.0%)	50	33 (80.5%)	8 (19.5%)	41	4 (8.0%)	46 (92.0%)	50
Stage									
Stages I	16 (29.1%)	39 (70.9%)	55	32 (82.1%)	7 (17.9%)	39	11 (20.0%)	44 (80.0%)	55
Stages II-IV	11 (22.0%)	39 (78.0%)	50	32 (82.1%)	7 (17.9%)	39	6 (12.0%)	44 (88.0%)	50

(OA: $n = 105$, EM: $n = 17$, BOT: $n = 9$, NE: $n = 28$

was 83.8% (88 cases). Among the TrkB-positive ovarian cancers, 88.5% (69 cases) were also BDNF positive (69 cases) (Table 2).

We then analyzed the molecular structure of the TrkB isoforms expressed in ovarian cancer. For the analyses, we selected samples (Table 1) after we confirmed TrkB protein expression by the above immunohistochemical analysis.

The RT-PCR results showed that the TGW neuroblastoma cell line expressed all domains of TrkB, as reported previously (*17*). The PCR products of this cell line were used as positive controls because the sequences of these products were consistent with the sequences of the TrkB domains in normal neural tissue registered in the NCBI database (NM006180.3/ variant a/TrkB, NM001007097.1/variant b/TrkB-T1, NM001018064.1/variant c/TrkB lacking exon 17, NM001018065.2/variant d/TrkB-T-Shc, and NM001018066.2/variant e/TrkB-T-Shc lacking exon 17). Typical RT-PCR results are shown in Figure 3. As a result, CCA and mucinous adenocarcinoma (MA) specimens lacked TrkB domains, as shown in Figure 4A, while all the specimens of serous and endometrioid adenocarcinoma expressed all domains of normal TrkB. In CCA, the absence of the TrkB-T-Shc (exons 18-19) and TrkB-T1 (exons 14-16) isoforms was observed with high frequency (9/21 and 6/21 cases, respectively). The percentages of these truncated isoforms decreased in parallel with the clinical stage progression (Figure 5). These results indicate that the decreased level of dominant-negative isoforms correlates with CCA malignancy. Other isoforms were also observed. Prominently, amplification of the exon 9-12 fragment, which includes the BDNF-binding site, was not observed (9/21 of CCA and 2/8 of MA) during the earlier stages. Other variants lacked the exon 5-9 fragment, which is detected only in silico, or the exon 14-18 fragment, which includes the Shc-binding

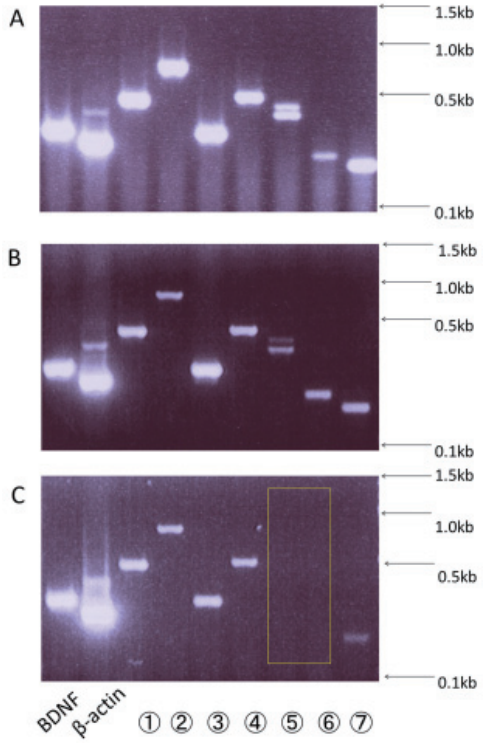

Figure 3. Semiquantitative RT-PCR analysis of ovarian cancer. (A) TGW cells (positive control). **(B)** Endometrioid adenocarcinoma (Icb) (patient number 18 in Figure 4). **(C)** Clear cell adenocarcinoma (Ica) (patient number 36 in Figure 4). The numbers below the panel indicate the PCR products shown in Figure 4.

domain. The absence of exons 18-19 (TrkB-T-Shc) was also observed in MA (1/8). The transmembrane domain (exon 12-14) and the TK domain (exon 22-23) were detected in all ovarian adenocarcinomas (OAs), suggesting the importance of these domains for TrkB expression. BDNF mRNA was expressed in all OAs, and no differences were observed in relation to the histology or clinical stage.

We then examined the expression of TrkB variants

A

	No	stage	① Exon5–9	② Exon9–12	③ Exon12–14	④ Exon14–16	⑤ Exon14–18	⑥ Exon18–19	⑦ Exon22–23	BDNF	β actin
mucinous	1	Ⅰa	■	■	■	■	■	■	■	■	■
	2	Ⅰa	■	■	■	■	■	□	■	■	■
	3	Ⅰa	■	■	■	■	■	■	■	■	■
	4	Ⅰa	■	□	■	■	■	■	■	■	■
	5	Ⅰca	■	■	■	■	■	■	■	■	■
	6	Ⅰca	■	□	■	■	□	■	■	■	■
	7	Ⅰcb	■	■	■	■	■	■	■	■	■
n=8	8	Ⅱc	■	■	■	■	■	■	■	■	■
serous	9	Ⅰca	■	■	■	■	■	■	■	■	■
	10	Ⅲb	■	■	■	■	■	■	■	■	■
	11	Ⅲc	■	■	■	■	■	■	■	■	■
	12	Ⅲc	■	■	■	■	■	■	■	■	■
	13	Ⅲc	■	■	■	■	■	■	■	■	■
	14	Ⅲc	■	■	■	■	*	■	■	■	■
	15	Ⅲc	■	■	■	■	■	■	■	■	■
n=8	16	Ⅳ	■	■	■	■	■	■	■	■	■
endometrioid	17	Ⅰa	■	■	■	■	■	■	■	■	■
	18	Ⅰcb	■	■	■	■	■	■	■	■	■
	19	Ⅱc	■	■	■	■	■	■	■	■	■
	20	Ⅱc/G1	■	■	■	■	■	■	■	■	■
	21	Ⅲb/G3	■	■	■	■	■	■	■	■	■
	22	Ⅲc/G2	■	■	■	■	■	■	■	■	■
	23	Ⅲc	■	■	■	■	*	■	■	■	■
	24	Ⅲc/G2	■	■	■	■	■	■	■	■	■
	25	Ⅲc/G3	■	■	■	■	■	■	■	■	■
n=10	26	Ⅳ/G3	■	■	■	■	*	■	■	■	■
clear cell	27	Ⅰa	■	□	■	■	□	■	■	■	■
	28	Ⅰa	■	■	■	■	■	■	■	■	■
	29	Ⅰa	■	■	■	■	*	■	■	■	■
	30	Ⅰc	■	■	■	■	■	■	■	■	■
	31	Ⅰc	■	□	■	■	■	■	■	■	■
	32	Ⅰc	■	□	■	□	■	■	■	■	■
	33	Ⅰc	■	■	■	■	■	■	■	■	■
	34	Ⅰc	■	■	■	■	■	■	■	■	■
	35	Ⅰc	■	□	■	□	□	□	■	■	■
	36	Ⅰca	■	■	■	■	□	□	■	■	■
	37	Ⅰcb	■	■	■	■	*	□	■	■	■
	38	Ⅰcb	■	■	■	■	*	□	■	■	■
	39	Ⅱc	■	□	■	□	□	□	■	■	■
	40	Ⅱcb	■	□	■	■	□	□	■	■	■
	41	Ⅲb	■	■	■	■	*	□	■	■	■
	42	Ⅲc	■	■	■	■	■	■	■	■	■
	43	Ⅲc	■	■	■	□	□	□	■	■	■
	44	Ⅲc	□	□	■	□	□	■	■	■	■
	45	Ⅳ	■	□	■	□	■	■	■	■	■
	46	rec	■	■	■	■	■	□	■	■	■
n=21	47	rec	■	□	■	■	□	■	■	■	■

B

	No	cell line	① Exon5–9	② Exon9–12	③ Exon12–14	④ Exon14–16	⑤ Exon14–18	⑥ Exon18–19	⑦ Exon22–23	BDNF	β actin
mucinous	1	TU-OM-1	■	■	■	■	■	■	■	■	■
	2	TU-OM-1/TX	■	■	■	■	■	■	■	■	■
	3	TU-OM-1/CDDP/TX	■	■	■	■	■	■	■	■	■
serous	4	HTOA	■	■	■	■	□	□	□	■	■
	5	HUOA	■	■	■	■	■	■	□	■	■
clear cell	6	HUOCA-Ⅱ	□	□	■	■	□	□	■	■	■
	7	W3UF	■	■	■	■	□	□	■	■	■
	8	ES-2	□	□	■	□	□	□	□	■	■

Figure 4. Summary of the expression of TrkB variants by ovarian adenocarcinomas and ovarian cancer cell lines. (A) Expression of TrkB variants by mucinous, serous, endometrioid, and clear cell adenocarcinomas. PCR products that were detected are shown in hatched boxes, and negative products are shown in open boxes. The asterisks in ⑤ indicate a single band (two PCR bands were observed in all other samples). **(B)** Expression of TrkB variants by mucinous, serous and clear cell adenocarcinoma cell lines. Boxes are the same as in **(A)**. TU-OM-1/TX and TU-OM-1/CDDP/TX indicate resistance to TX and CDDP/TX, respectively.

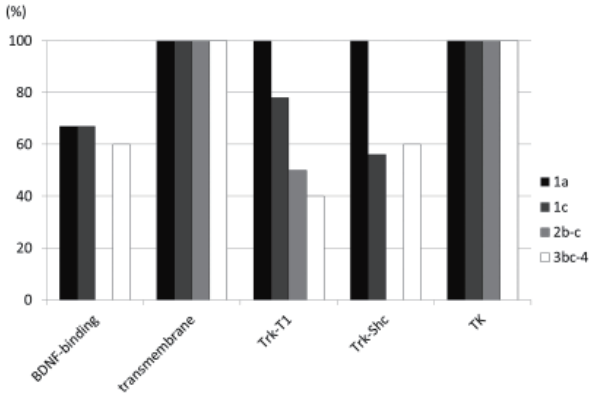

Figure 5. Expression of the TrkB domain by the clinical stage of CCA. CCA samples were categorized in 4 stages (1a: black bar, 1c: dark gray bar, 2b-c: light gray bar and 3bc-4: white bar) (n = 21), and the expression ratio (%) of each domain (BDNF-binding site; exon9-12, transmembrane site; exon15, TrkB-T-Shc tail; exon18-19 and TrkB-T1; exon14-16) in the 21 CCA specimens is shown in the histograms. The transmembrane domain and TK tail were expressed in all stages. The expression of the TrkB-T1 tail and TrkB-T-Shc tail (%) decreased with clinical stage progression.

in ovarian cancer cell lines. Similar to the findings in clinical specimens, the CCA cell lines (HUOCA-II, W3UF, and ES-2) lacked the TkB-T-Shc and TrkB-T1 isoforms. The two serous adenocarcinoma cell lines (HTOA and HUOA) expressed either the TrkB-T-Shc or TrkB-T1 isoform. These results suggest that the cell lines partially maintained the TrkB isoform expression characteristics of the parental tumors (Figure 4B).

To confirm the structure of the TrkB mRNA, its cDNA sequence was analyzed. In 13/23 of tumors (2/5 MAs, 1/5 serous, 6/7 endometrioid, and 4/6 CCA), the sequence was the same as that of normal TrkB (NTrk2-NM006180), with the exception of several single nucleotide polymorphisms, suggesting that large mutations such as a frame shift did not occur at the exon level in these cancer cell lines (data not shown).

4. Discussion

TrkB, which promotes cell survival and EMT, is over-expressed in OA. Using immunohistochemistry, we demonstrated that TrkB protein expression was increased at the invasive edge (Figure 2B). However, as TrkB possesses many isoforms, including dominant negative isoforms, the overall signal level has not been clearly evaluated in association with the progression of OAs. Our results first demonstrated that although TrkB transcripts are observed in all ovarian cancer tissues, the mRNA expression of the truncated TrkB isoforms (TrkB-T-Shc and TrkB-T1) is decreased in CCA. Because these truncated isoforms are suggested to have a dominant-negative effect, their absence may indicate the augmentation of the TrkB TK signal in CCA tumor cells.

As depicted in Figure 1, the structure of the TrkB gene includes many functional domains. The first five exons of TrkB serve as alternative transcription start sites (*11*). Exons 5-14 encode the extracellular domain that contains a signaling sequence for membrane localization as well as post-translationally glycosylated cysteine-rich and leucine-rich regions and two immunoglobulin-like (IG-like) domains (*18*). Exon 12, exon 15, and exons 20-24 encode the binding site for neurotrophins (*19*), the transmembrane domain, and the intracellular TK domain (*20*), respectively. While there is a possibility that TrkB could have more than 100 isoforms, only three isoforms, including full-length TrkB, TrkB-T-Shc and TrkB-T1, have been identified as major splice variants in previous studies. The two isoforms are mainly observed in normal neuronal tissues (*11*) and other tissues (*20*). The present analysis revealed that a significant number of CCA specimens and some MA specimens or cell lines lacked the truncated splice variants TrkB-T-Shc and TrkB-T1 (Figures 4A and 4B). The intracellular domain of full-length TrkB contains an Shc-binding domain, a TK domain and a PLC-γ-binding domain (*2*). While these motifs are necessary for the normal signal transduction of TrkB (*2*), the expression of truncated isoforms may alter the signaling cascade in cancer cells. For example, TrkB-T-Shc and TrkB-T1 suppress the activation of full-length TrkB, and the lack of these truncated isoforms may therefore augment full-length TrkB activity, leading to the suppression of anoikis and TrkB-T1 functions such as regulation of the cytoskeleton or BDNF secretion (*21*). Because CCA and MA are known to be more malignant than other types of ovarian cancer, loss of the dominant-negative effect of TrkB may promote EMT, which has been correlated with the characteristics of CCA.

We also found an extracellular domain variant lacking the amplification of exons 9-12 in ovarian cancer. Because this region contains the binding site for BDNF, further analysis of whether aberrant splicing of exons 9-12 influences BDNF binding is required. If this splice variant lacking exon 9-12 amplification is a constitutively active molecule that transmits signals independent of BDNF binding, there is a possibility that it could promote CCA. On the other hand, even if the BDNF binding site is intact in the region, irregular TrkB autocrine signals may be induced. These irregular TrkB signals may help to induce EMT-promoting transcription factors such as Twist-1 and Snail-1/2 in TrkB-over-expressed cancer tissues (*6*).

It has already been reported that BDNF is abundantly expressed in gynecological tissues (*4*), and we found that BDNF was highly expressed in almost all of the gynecological tissues that we analyzed, including ovarian cancers, benign ovarian tumors, and normal endometrium (data not shown). As BDNF promotes TrkB expression (*22*), autocrine activation of TrkB in ovarian tissues may support malignant characteristics when BDNF secretion

is augmented in the absence of dominant-negative isoforms. Our results indicate that the expression of irregular TrkB variants may be a potential marker of OA malignancy and a target of CCA treatment. The effects of TrkB isoform expression on EMT in ovarian cancer should be clarified.

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