Tissue factor pathway inhibitor-2 is specifically expressed in ovarian clear cell carcinoma tissues in the nucleus, cytoplasm and extracellular matrix

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Abstract. Tissue factor pathway inhibitor-2 (TFPI-2) is a promising candidate as a serum biomarker of ovarian clear cell carcinoma (OCCC), a lethal histological subtype of epithelial ovarian cancer (EOC). TFPI-2 is a secreted serine protease inhibitor that suppresses cancer progression through the inhibition of matrix protease activities. Previous studies have also identified TFPI-2 in the nucleus, and a possible function of nuclear TFPI-2 as a transcriptional repressor of matrix metalloproteinase-2 (MMP-2) was recently demonstrated. We are currently establishing TFPI-2 as a serum biomarker for OCCC patients; however, TFPI-2 expression in OCCC tissues has not been previously investigated. In the present study, we examined TFPI-2 expression and its localization in 11 OCCC cell lines by western blotting and enzyme-linked immune assay. Four cell lines expressed TFPI-2 in the nucleus, cytoplasm and culture plate–attached extracellular fraction, while four other cell lines expressed TFPI-2 only in the extracellular fraction. In the remaining three cell lines, TFPI-2 was not identified in any fraction. The amount of secreted soluble TFPI-2 showed similar trends to that of the plate-attached fraction. We next investigated the expression levels and distribution of TFPI-2 in surgically resected EOC tissues by immunohistochemistry. In 52 of the 77 (67.5%) OCCC tumors, TFPI-2 expression was detected in at least one of the nuclear, cytoplasmic and extracellular matrix fractions. In contrast, we did not identify TFPI-2 in the other EOC subtypes (n=65). TFPI-2-positive expression distinguished CCC from the other EOC tissues with a sensitivity of 67.5% and specificity of 100%. Although the inherent tumor suppressor function, statistical analyses failed to demonstrate correlations between TFPI-2 expression and clinical parameters, including 5-year overall survival, except for the patient age. In conclusion, we identified TFPI-2 expression in the nucleus, cytoplasm and extracellular matrix in OCCC tissues. The high specificity of TFPI-2 may support its use for diagnosis of OCCC in combination with existing markers.

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Abbreviations: TFPI-2, tissue factor pathway inhibitor-2; OCCC, ovarian clear cell carcinoma; EOC, epithelial ovarian cancer; CA125, cancer antigen 125; PBS, phosphate-buffered saline; WCF, whole cell fraction; ECF, extracellular fraction; ECM, extracellular matrix; CM, conditioned medium; AIA, automated immunoassay analyzer; KCCH, Kanagawa Cancer Center Hospital; FFPE, formalin-fixed and paraffin-embedded; IHC, immunohistochemistry

Key words: TFPI-2, ovarian clear cell carcinoma, immunohistochemistry, biomarker, subcellular localization

Introduction

Ovarian cancer is the most lethal gynecological malignancy in developed countries (1). In 2018, approximately 295,400 new cases of ovarian cancer were diagnosed and 184,800 patients with ovarian cancer died worldwide (2). Clear cell carcinoma (CCC) is one of the common histological types of epithelial ovarian cancer (EOC) (3). The frequency of ovarian CCC (OCCC) varies depending on ethnicity; CCC accounts for 11.7-26.9% of Japanese EOC cases in comparison with 4.6-8.4% of EOC in North America (4,5). About half of the OCCC cases are diagnosed at stage I and have a good prognosis (6). However, advanced stage or recurrent OCCC cases have worse prognosis than the other EOC subtypes due to the resistance to standard platinum-based chemotherapy (7). Therefore, early detection and complete resection are crucial in OCCC treatment. Cancer antigen 125 (CA125) is currently the most frequently used serum biomarker for EOC. However, CA125 is also elevated in benign conditions such as endometrial cyst and peritonitis, menstruation and other intra-abdominal

malignancies (8). Thus, CA125 does not necessarily distinguish malignancy. Additionally, CA125 often fails to detect OCCC even at advanced stages (9).

Tissue factor pathway inhibitor-2 (TFPI-2) protein, a homologue of tissue factor pathway inhibitor (TFPI), is a secreted protease inhibitor containing an N-terminal signal peptide and Kunitz-type serine protease inhibitory domains (10). Despite its structural similarity to TFPI, TFPI-2 has weak inhibitory activity against the tissue factor blood coagulation pathway, which is initiated by the serine protease tissue factor-coagulation factor VIIa complex, and instead inhibits a wide variety of serine proteases, such as plasmin, plasma kallikrein, trypsin and chymotrypsin (10). TFPI-2 is predominantly and highly expressed in placenta (11,12). Although several studies have examined the association between TFPI-2 and preeclampsia (13,14), the biological function of TFPI-2 is not fully understood.

Many reports have shown that TFPI-2 is genetically silenced in aggressive cancers, such as glioma (15), non-small cell lung cancer (16), pancreatic cancer (17), breast cancer (18), malignant melanoma (19) and hepatocellular carcinoma (20), indicating its tumor-suppressor character. The anticancer functions of TFPI-2 are generally thought to be mediated by its protease inhibitory activities, which lead to inhibition of cell proliferation, invasion or angiogenesis and augmentation of apoptosis (21,22). Recent studies also suggest another tumor-suppressor aspect of TFPI-2, demonstrating that exogenously applied TFPI-2 localized in the nucleus of fibrosarcoma cells (23) and overexpressed TFPI-2 in breast cancer cells negatively regulate matrix metalloproteinase-2 (MMP-2) expression (24).

In contrast to the results showing epigenetic silencing of TFPI-2 in several tumor types, we recently reported that cultivated OCCC cells produce and secrete TFPI-2 into medium and we initiated studies to develop TFPI-2 as a specific serum biomarker for preoperative clinical diagnosis for OCCC (25,26). Serum TFPI-2 level discriminated CCC from other histological types of EOC and endometrial cyst (26), which is a risk factor for CCC (27). Although we are considering that serum TFPI-2 is derived from OCCC tumor cells, TFPI-2 expression was also reported in endothelial cells, which are distributed throughout the body (23). Furthermore, non-secreted fractions of TFPI-2 were reported in *in vitro* studies in other tumor types. Therefore, in the present study, we examined TFPI-2 expression and localization of TFPI-2 in multiple OCCC cell lines and in surgically removed OCCC tissues including tissues of other EOC histologic types. We also investigated the association between TFPI-2 expression and clinical characteristics of OCCC patients to clarify the role of TFPI-2 in OCCC.

Materials and methods

Cell lines and cell culture. The OCCC cell lines ES-2 (ATCC CRL-1978) and TOV-21G (ATCC CRL-11730) were purchased from the American Type Culture Collection. OVISE (JCRB1043), OVMANA (JCRB1045), OVTOKO (JCRB1048), RMG-1 (JCRB0172) and HAC-2 (JCRB1359) cells were obtained from JCRB Cell Bank. JHOC-5 (RCB1520), JHOC-7 (RCB1688), JHOC-8 (RCB1723) and JHOC-9 (RCB2226) cell lines were from RIKEN Bioresource Center Cell Bank. These OCCC cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37˚C in a humidified atmosphere of 5% CO_2.

Preparation of subcellular fractions. Cells were cultured for 2 days in 100-mm plates until they reached semi-confluency. Cells were washed with phosphate-buffered saline (PBS) and then dissociated using Accutase reagent (Nacalai Tesque) according to the manufacturer's instruction. Dissociated cells were collected to prepare the whole cell fraction (WCF). Plates were rinsed twice with PBS, and the fraction that remained attached to the plate was collected by scraping the plates with lysis buffer and was considered the extracellular fraction (ECF). (NuPAGE NP0007, Thermo Fisher Scientific, Inc.). The Nuclear Extract Kit (Active Motif Inc.) was used for preparation of cytoplasmic and nuclear fractions from WCFs according to the manufacturer's instructions. Cells were cultured with 10 ml of RPMI-1640 medium supplemented with 10% FBS and penicillin-streptomycin for 2 days in 100-mm plates. Culture medium of semi-confluent cells was collected and centrifuged at 180 x g for 3 min. The supernatant was obtained as conditioned medium (CM).

Western blotting. Western blotting was performed using the NuPAGE 4-12% gradient Bis-Tris Protein Gel system (Thermo Fisher Scientific, Inc.) with MOPS running buffer (Thermo Fisher Scientific, Inc.). To detect TFPI-2, we used mouse monoclonal anti-TFPI-2 antibody (clone 28Aa, 1 μg/ml, diluted 1:2,000) raised against a synthetic peptide antigen corresponding to the N-terminal of mature TFPI-2 protein after cleavage of the putative signal peptide (13). Anti-vinculin (V9131, diluted 1:10,000, Sigma-Aldrich; Merck KGaA), anti-Lamin A (sc-20680, diluted 1:500, Santa Cruz Biotechnology, Inc.) and anti-α-tubulin antibodies (T-9026, diluted 1:3,000, Sigma-Aldrich; Merck KGaA) were used for protein loading controls. Secondary antibody reaction was performed with peroxidase-conjugated anti-mouse IgG (NA931, 1:100,000, Cytiva) or anti-rabbit IgG (NA934, 1:100,000, Cytiva). Detection was performed using the ImmunoStar LD enhanced chemiluminescence detection reagent (FUJIFILM Wako Chemicals).

TFPI-2 concentration in CM. The TFPI-2 concentration in CM was measured on an automated immunoassay analyzer (AIA) system (TOSOH, Japan) as described previously (26). Briefly, measurement of TFPI-2 using the AIA system was completed as a sandwich-type, one-step immune fluorometric assay using two different anti-TFPI-2 monoclonal antibodies, one of which was coated on magnetic beads and the other was labeled with alkaline phosphatase. As the calibration standard of the assay, recombinant TFPI-2 protein was prepared from the CM of SP2/0 cells transfected with the TFPI-2 expression vector and spiked into sample dilution buffer.

Patients and sample collection. A total of 142 patients with a confirmed histopathological diagnosis of EOC at Kanagawa Cancer Center Hospital (KCCH), Japan were included in this study. Patients who underwent treatment before

primary debulking surgery or exploratory laparotomy were excluded. Patients with other cancers were also excluded. We examined all 71 EOC patients who matched the criteria from 2014 to 2017 to evaluate the expression of TFPI-2 along with the histological subtypes. Due to the small number of the included cases, 8 patients with endometrioid carcinoma and 14 patients with mucinous carcinoma were selected from the period before 2014 and additionally examined. Formalin-fixed and paraffin-embedded (FFPE) tissue sectioned to 4 μm-thickness were prepared from archives of the Department of Pathology, KCCH. Whole tissue sections of tumors of all enrolled patients were analyzed. Representative non-neoplastic regions of the surgical specimens of EOC cases were also examined in 18 cases, including endometrium and fallopian tubal epithelium (CCC: 9, serous: 3, endometrioid: 3, mucinous: 3). Written informed consent for research using specimens derived from routine clinical procedures was obtained from all patients. The experimental protocol of the present study was reviewed and approved by the Institutional Review Board of KCCH (approval no. Ethics-2018-10).

Immunohistochemical analysis of TFPI-2 expression. FFPE tissue specimens on glass slides were routinely stained with hematoxylin and eosin. Deparaffinized and rehydrated slides were immersed in 0.01 M citrate, pH 6.0 (Sigma-Aldrich; Merck KGaA), and heat-induced antigen retrieval was performed in an autoclave at 110˚C for 15 min. Slides were cooled to room temperature, washed in PBS and immersed in 3% H_2O_2 diluted in methanol. For primary antibody, 28Aa antibody was diluted to 5 μg/ml. Histofine Simplestain Max PO (M) (Nichirei) and Histofine DAB Substrate kit (Nichirei) were used to detect the labeled antigens. Placental tissue was used as positive control for TFPI-2 staining (13). Non-specific mouse IgG was used as a negative control. We conducted an absorption test to evaluate the specificity of the staining. Antibodies were incubated with a 20-fold excess molar concentration of the antigen for 24 h prior to the primary antibody reaction (28). The antigen for the 28Aa antibody is the 14 amino acid residues corresponding to the N-terminus of mature TFPI-2 protein, NH2-DAAQEPTGNNAEIC-COOH (13), linked to keyhole limpet hemocyanin. We used another anti-TFPI-2 antibody B-7 (sc-48380, diluted 1:40, Santa Cruz Biotechnology, Inc.) for detection of nuclear TFPI-2. The B-7 antibody is a mouse monoclonal antibody that was raised against peptides corresponding to amino acid residues 71-190 of human TFPI-2. We also conducted an absorption test using placental tissue with recombinant full-length TFPI-2 protein (OriGene) as antigen. TFPI-2 protein staining (cytoplasmic and nuclear staining) was scored by the H-score method (29). Briefly, the H-score was calculated as the sum of the products of multiplying the staining intensity (0, 1+, 2+, 3+) by percentage stained area. For example, in a case with the intensity and percentage staining of 0+: 70%, 1+: 20%, 2+: 10% and 3+: 0%, the H-score is calculated as 40 (40=0x70 + 1x20 + 10x2 + 0x3). Under a pathologist supervision, automated scoring on tumor regions was performed using Aperio's annotation software 'Aperio Cytoplasm Algorithm' (Leica Biosystem). We defined the cut-off value for TFPI-2 positivity as an H-score of 1 to reduce false negatives. We evaluated TFPI-2 expression within extracellular matrix (ECM) as 'positive' or 'negative.' We analyzed TFPI-2 expression and clinical characteristics of the OCCC patients.

Statistical analysis. Statistical analysis was performed using IBM SPSS Statistics 19 software (IBM Corp.). Clinicopathological parameters were evaluated using Kruskal-Wallis test or Mann-Whitney U test for continuous variables and Fisher's exact test for non-continuous variables. Relationships between TFPI-2 expression and 5-year overall survival were estimated by Kaplan-Meier method and compared by log rank test. Cox regression analysis was used for multivariate analysis of 5-year overall survival. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression, subcellular localization and secretion of TFPI-2 in OCCC cell lines. Western blotting using the monoclonal anti-TFPI-2 28Aa antibody (13) revealed that TFPI-2 was expressed in 8 out of the 11 CCC cell lines examined (Fig. 1A). All eight cell lines showed TFPI-2 expression in ECF and four cell lines also expressed TFPI-2 in the WCF. In all cell lines, TFPI-2 was much more abundant in ECF than in WCF. We next fractionated TFPI-2 containing WCFs of the four cell lines into nuclear and cytoplasmic fractions. TFPI-2 was detected in both cytoplasmic (CP) and nuclear fractions (NE) (Fig. 1B). TFPI-2 polypeptides of three molecular weights (27, 31, 33 kDa) (12) were observed in all 3 fractions, but the larger two molecules were predominant (Fig. 1B). Three cell lines did not express TFPI-2 in any fraction. We also examined TFPI-2 concentration in CM (Fig. 1C). The amount of secreted TFPI-2 in the CM was generally correlated to the levels in ECF. RMG-1 and OVMANA cells strongly expressed TFPI-2 in ECF by western blotting. In contrast, TFPI-2 concentration was high in CM in RMG-1 cells but low in OVMANA cells.

Immunohistochemical analysis of TFPI-2 expression in surgically removed EOC tissues. FFPE samples prepared from 142 patients including 77 OCCC and 65 non-CCC EOC cases were subjected to immunohistochemistry (IHC). The patient clinical information is shown in Table I. The mean age of patients at surgery was 57 years (range 36-84 years).

Experiments with placental tissue confirmed that the antibody stained the cytoplasm of syncytiotrophoblasts, as reported previously (13) (Fig. 2A). We confirmed the specificity of the antibody by an absorption test using the immunized antigen for the 28Aa antibody (Fig. 2B). IHC revealed TFPI-2 in the cytoplasm of tumor cells and in the ECM of OCCC tissues (Fig. 2C and D). We did not detect any nuclear TFPI-2 staining using the 28Aa antibody. Therefore, we next assessed the localization of TFPI-2 using another TFPI-2 antibody (B-7). We confirmed that the B-7 antibody also stained the cytoplasm of syncytiotrophoblasts in placental tissue (Fig. 3A). The specificity of the B-7 antibody was confirmed by absorption test (Fig. 3B). We detected TFPI-2 both in the nucleus and cytoplasm with the B-7 antibody (Fig. 3C); however, signals in ECM were weaker than in staining with the 28Aa antibody (Figs. 2D and 3D). Therefore, we decided to use the B-7 antibody to evaluate nuclear and cytoplasmic expression of TFPI-2, while the 28Aa antibody was used to evaluate TFPI-2 expression in ECM.

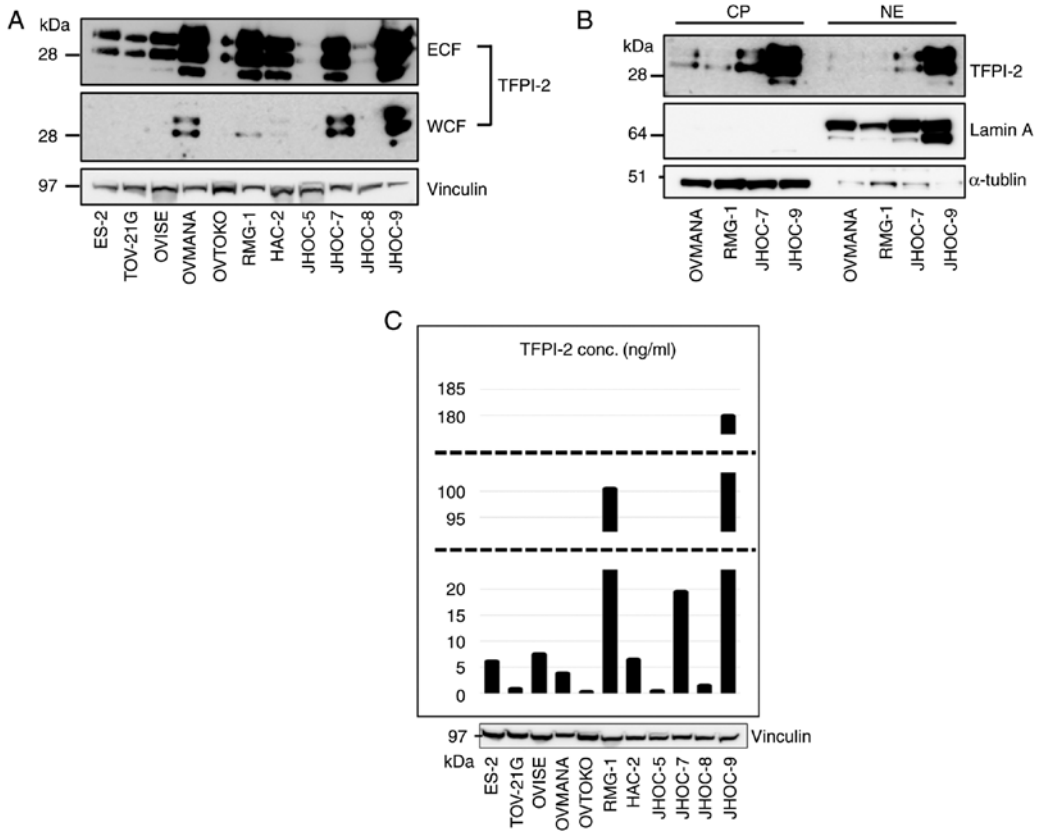

Figure 1. Western blotting and enzyme-linked immune assay of TFPI-2. (A) Western blotting of TFPI-2 expression in 11 cell lines. Vinculin was used for WCF loading control. (B) WCFs were further divided into nuclear and cytoplasm fractions. Lamin A and α-tubulin were used for nucleus and cytoplasm markers, respectively. (C) TFPI-2 concentration in CM was assessed by enzyme-linked immune assay. TFPI-2, tissue factor pathway inhibitor-2; ECF, extracellular fraction; WCF, whole cell fraction; NE, nuclear extract; CP, cytoplasm; CM, conditioned medium.

The H-score method using automated scoring software was applied to evaluate TFPI-2 staining (Fig. S1). The H-scores and staining categorization of EOC tissues are shown in Table II. Among OCCC cases, 52/77 (67.5%) specimens were positive for TFPI-2; among these samples, 35/77 (45.5%) showed cytoplasmic staining, 10/77 (13.0%) showed nuclear staining and 35/77 (45.5%) showed staining in ECM (shown as a Venn diagram in Fig. S2). All cases with positive nuclear staining also showed positive staining in the cytoplasm, and 7/77 (9.1%) cases showed positive staining in all three fractions (Fig. S2). In contrast, TFPI-2 was not detected in any of the non-CCC cases (Fig. 4A-C). TFPI-2 expression levels evaluated by IHC distinguished CCC from non-CCC with 67.5% sensitivity and 100% specificity. Previous studies showed that TFPI-2 is expressed in endometrium (30,31). Therefore, we next performed IHC for the non-tumor samples using B-7 antibody in the same manner. Out of 18 cases, 17 cases were negative for TFPI-2 in endometrium cells (Fig. 5A). In one case (5.6%), endometrium cells were focally positive for TFPI-2. Fallopian tube epithelial cells were all negative for TFPI-2 expression (Fig. 5B).

We next statistically analyzed the correlations between TFPI-2 cytoplasmic expression and clinicopathological characteristics of the OCCC patients according to previous studies (32,33). We examined patient age, parity, menopausal status, rate of elevated serum CA125 level (>35 U/ml) and distribution of cancer stage (FIGO: International Federation of Gynecology and Obstetrics staging and TNM classification) in univariate analysis according to the cytoplasmic expression status for TFPI-2 (Table III). The median patient age was significantly younger for patients positive for TFPI-2 than for patients negative for TFPI-2 (56 vs. 60.5 years, respectively; P=0.019). Parity, menopausal status, rate of elevated serum level of CA125, FIGO and TNM staging did not significantly correlate with TFPI-2 expression. Kaplan-Meier analysis showed that the 5-year overall survival was not significantly affected by TFPI-2 expression (P=0.621, log-rank test) (Fig. S3A). Multivariate analysis revealed that TFPI-2 expression was not an independent prognostic factor (Table SI). Analyses with nuclear and ECM TFPI-2 expression showed similar results (Fig. S3B-D, Tables SI-SIV).

Discussion

In the present study, we found that tissue factor pathway inhibitor-2 (TFPI-2) is expressed in surgically removed ovarian clear cell carcinoma (OCCC) tissues. We previously identified TFPI-2 as a CCC biomarker using secretome-based analysis of CM derived from OCCC cell lines (25,26) and reported that TFPI-2 may be a useful serum biomarker for OCCC patients. The confirmation of TFPI-2 expression in OCCC tumor cells in surgical tissues using IHC strongly supports the development of TFPI-2 as a serum tumor biomarker.

We demonstrated that TFPI-2 is localized in the nucleus as well as the cytoplasm and extracellular fraction (ECF)

Table I. Clinicopathological characteristics of the 142 epithelial ovarian cancer patients.

Characteristics	OCCC (n=77)	SC (n=20)	EMC (n=19)	MOC (n=17)	Others (n=9)	P-value
Period (year)	2005 to 2017	2014 to 2017	2011 to 2017	2005 to 2017	2014 to 2017	
Age in years, median (range)	58 (36-75)	67.5 (37-80)	54 (38-83)	55 (38-84)	60 (47-83)	P=0.0875
Parity (%)						P=0.024
No (0)	36 (46.8)	4 (20.0)	7 (36.8)	3 (17.6)	6 (66.7)	
Yes (≥1)	41 (53.2)	16 (80.0)	12 (63.1)	14 (82.4)	3 (33.3)	
Menopausal status (%)						P=0.149
Premenopause	18 (23.4)	2 (10.0)	7 (36.8)	7 (41.2)	3 (33.3)	
Postmenopause	59 (76.6)	18 (90.0)	12 (63.1)	10 (58.8)	6 (66.7)	
CA125 (%)						P=0.321
<35	24 (31.2)	2 (10.0)	7 (36.8)	5 (29.4)	2 (22.2)	
≥35	53 (68.8)	18 (90.0)	12 (63.1)	12 (70.6)	7 (77.8)	
FIGO (%)						P<0.001
I/II	61 (79.2)	3 (15.0)	17 (89.4)	16 (94.1)	5 (55.6)	
III/IV	16 (20.8)	17 (85.0)	2 (10.5)	1 (5.9)	4 (44.4)	
Site of specimen (%)						P<0.001
Primary site	77 (100)	17 (85.0)	19 (100)	17 (100)	7 (77.8)	
Omentum	0 (0)	3 (15.0)	0 (0)	0 (0)	2 (22.2)	

OCCC, ovarian clear cell carcinoma; SC, serous carcinoma; EMC, endometrioid carcinoma; MOC, mucinous ovarian carcinoma. Others: Two carcinosarcoma, one squamous cell carcinoma arising from mature cystic teratoma, one small cell carcinoma, one adenofibrocarcinoma, one mixed epithelial tumor, one large cell neuroendocrine carcinoma, one undifferentiated and one adenocarcinoma (not otherwise specified). Kruskal-Wallis test was used for age. Fisher's exact test was used for other parameters. CA125, cancer antigen 125; FIGO, Federation of Gynecology and Obstetrics.

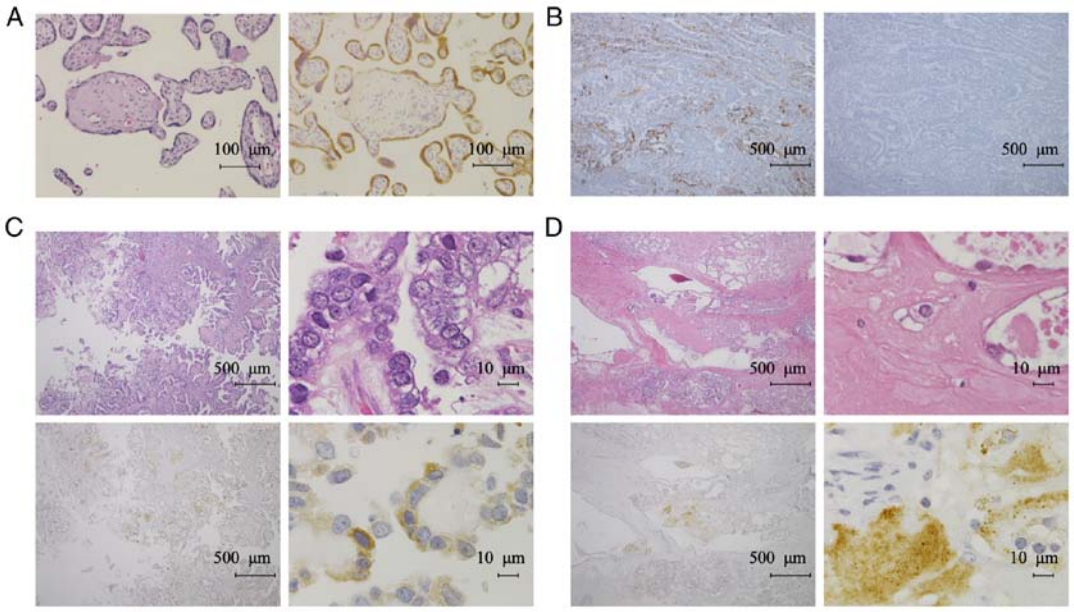

Figure 2. IHC analysis of TFPI-2 subcellular localization. (A) Typical H&E staining (left) and TFPI-2 staining using the 28 Aa antibody (right) in placenta specimens. (B) Specificity of TFPI-2 antibody was confirmed in an OCCC specimen by absorption test using the 14 amino acid peptide antigen corresponding to the N-terminus of mature TFPI-2 protein. Left: No antigen control. Right: Experiment with pre-absorbed antibody. (C) Typical H&E staining (top) and cytoplasmic TFPI-2 staining (bottom) patterns in an OCCC specimen. (D) Typical H&E staining (top) and TFPI-2 staining (bottom) patterns in a stromal lesion of an OCCC specimen. IHC, immunohistochemistry; TFPI-2, tissue factor pathway inhibitor-2; H&E, hematoxylin and eosin; OCCC, ovarian clear cell carcinoma.

of cultivated OCCC cells. TFPI-2 has been characterized as a secreted protein (23) that contains a signal peptide at its N-terminus, and mature TFPI-2 protein is secreted into the ECF through the endoplasmic reticulum and secretory pathway (11,34). A recent study, however, showed that TFPI-2 was also localized in the nucleus and cytoplasm in

Table II. TFPI-2 expression score according to subcellular localization.

Subcellular localization	H-score	CCC (n=77) n (%)	Non-CCC (n=65) n (%)
Nuclear			
Negative	0	67 (87.0)	65 (100)
Positive	1-9	1 (1.3)	0 (0)
	10-29	4 (5.2)	0 (0)
	30-	5 (6.5)	0 (0)
Cytoplasm			
Negative	0	42 (54.5)	65 (100)
Positive	1-9	20 (26.0)	0 (0)
	10-29	9 (11.7)	0 (0)
	30-	6 (7.8)	0 (0)
ECM			
Negative		42 (54.5)	65 (100)
Positive		35 (45.5)	0 (0)

Cut-off for positive/negative expression is H-score=1. TFPI-2, tissue factor pathway inhibitor-2; CCC, clear cell carcinoma; ECM, extracellular matrix.

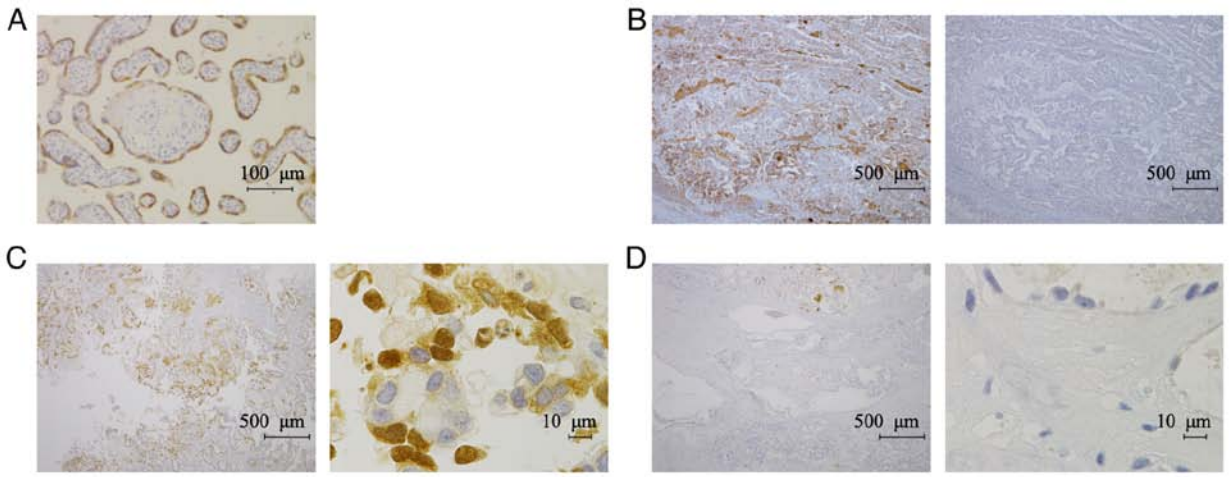

Figure 3. IHC analysis of TFPI-2 using the B-7 antibody. (A) Typical TFPI-2 staining in a placenta specimen using the B-7 antibody. TFPI-2 was detected in the syncyiotrophoblasts. (B) B-7 antibody specificity was confirmed in an OCCC specimen by absorption test using recombinant full-length TFPI-2 protein as an antigen. Left: No antigen control. Right: Experiment with pre-absorbed antibody. (C) B-7 antibody stains the nucleus and cytoplasm in the same sample shown in Fig. 2C. (D) B-7 antibody failed to stain ECM in the same sample shown in Fig. 2D. IHC, immunohistochemistry; TFPI-2, tissue factor pathway inhibitor-2; OCCC, ovarian clear cell carcinoma; ECM, extracellular matrix.

endothelial cell lines (23), and TFPI-2 exogenously added to culture medium *in vitro* was rapidly internalized and distributed in both nucleus and cytoplasmic fractions. A nuclear localization signal was found in the C-terminal tail of TFPI-2 (23). In the nucleus, TFPI-2 regulates MMP-2 gene transcription through the interaction with AP-2a, a transcription factor important for the expression of many genes (24). In the cytoplasm, TFPI-2 regulates ERK signaling and interacts with a-actinin-4 and myosin-9, resulting in increased cancer cell activities (35). Consistent with the *in vitro* study, we confirmed the nuclear, cytoplasm, and extracellular matrix (ECM) subcellular localization of TFPI-2 in surgically resected OCCC tissues. We detected TFPI-2 mainly in the ECF *in vitro*; however, the four cell lines with the highest expression of TFPI-2 also expressed TFPI-2 in both the nucleus and cytoplasm. Three different molecular sized TFPI-2 polypeptides, which are speculated to be derived from differential glycosylation events (12), were detected in all three fractions. Taken together, these findings suggest that mature TFPI-2, after cleavage of the signal peptide and posttranslational modifications, might be retained in the cytoplasm or internalized after secretion and distributed into the cytoplasm or nucleus when large amounts of TFPI-2 are produced. In OCCC OVMANA cells, the level of secreted TFPI-2 was not as high as its expression in ECF. In contrast, the majority of secreted TFPI-2 in ES-2 cells seemed to be retained in the medium. The mechanisms regulating TFPI-2 localization remain to be elucidated.

Table III. Clinicopathological characteristic and TFPI-2 cytoplasmic expression in 77 CCC samples.

Characteristics	Negative (n=42)	Positive (n=35)	P-value
Age in years, median (range)	60.5 (36-74)	56 (39-75)	P=0.019
Parity, n (%)			
No (0)	20 (47.6)	16 (45.7)	
Yes (≥1)	22 (52.4)	19 (54.3)	P=0.990
Menopausal status, n (%)			
Premenopause	7 (16.7)	11 (31.4)	
Postmenopause	35 (83.3)	24 (68.6)	P=0.177
CA125 (U/ml), n (%)			
<35	13 (31.0)	11 (31.4)	
≥35	29 (69.0)	24 (68.6)	P=0.990
FIGO, n (%)			
I/II	32 (76.2)	29 (82.9)	
III/IV	10 (23.8)	6 (17.1)	P=0.577
pT			
pT1/2	33 (78.6)	29 (82.9)	
pT3	9 (21.4)	6 (17.1)	P= 0.775
pN			
pN0	8 (19.0)	4 (11.4)	
pN1	1 (2.4)	0 (0)	
pNx	33 (78.6)	31 (88.6)	P=0.441
M			
M0	41 (97.6)	33 (94.3)	
M1	1 (2.4)	2 (5.7)	P=0.588

Mann-Whitney U test was used for continuous variables. Fisher's exact test was used for non-continuous variables. TFPI-2, tissue factor pathway inhibitor-2; CCC, clear cell carcinoma; CA125, cancer antigen 125; FIGO, Federation of Gynecology and Obstetrics.

In this study, we demonstrated the specificity of TFPI-2 for CCC in IHC. CCC is pathologically diagnosed based on morphologic features such as hobnail cells with clear cytoplasm (3). However, tumors containing clear cells with heterogeneous features are not reproducibly diagnosed (3). Currently, hepatocyte nuclear factor-1β (HNF-1β) immunohistochemical expression (sensitivity, 82.5-85.2%; specificity, 76.5-95.2%) (36,37), Napsin A (38) and glypican-3 (39) are candidates for CCC IHC markers. In this study, we showed that TFPI-2 was only identified in CCC tissues and not in non-CCC EOC tissues. This result is well consistent with The Human Protein Atlas data, which examined TFPI-2 expression in limited numbers of EOC surgical specimens by IHC but did not detect any cases with positive TFPI-2 expression (serous 0/5, mucinous 0/4, endometrioid 0/2 cases; CCC cases were not enrolled) (40). Our results showed that TFPI-2 expression distinguished CCC from non-CCC with a sensitivity of 67.5% and specificity of 100%. The high specificity of TFPI-2 may support its use for diagnosis of OCCC in combination with existing markers. We propose TFPI-2 as an IHC biomarker for histopathological diagnostics as well as serum biomarker for OCCC patients.

We found that all serous carcinoma cases in the current study group were negative for TFPI-2 in IHC. We previously showed that serum TFPI-2 levels greater than 345 pg/ml can pre-operatively discriminate OCCC from other EOC subtypes and borderline ovarian tumors with a sensitivity of 71.4% and specificity of 85.7% (25,26). Additionally, we found that serum TFPI-2 level was also increased in 29.4% of serous carcinoma patients (26). In this study, all serous carcinoma cases were negative for TFPI-2 despite setting the H-score cut-off value very low. Considering our IHC results, we speculate that the elevation of TFPI-2 in the serum of serous carcinoma patients was derived from non-tumor cells such as endothelial cells (23) or platelets (41), although the numbers of examined serous carcinoma cases were limited and the putative mechanisms are currently unclear.

We then examined the clinical significance of TFPI-2 expression in OCCC tissues but did not identify any significant association between TFPI-2 expression in the primary site and aggressiveness of the OCCC cases. This is not consistent with published data from other cancer types, which showed that low expression of TFPI-2 in IHC is associated with poor survival in breast and pancreatic cancer patients (32,33). The tumor suppressor-like activity of TFPI-2 suggested by these reports are consistent with *in vitro* and animal experiments showing that secreted TFPI-2 reduces invasiveness, through preventing ECM degeneration by inhibiting proteases, such as plasmin or MMPs (42,43). In many cancer types, TFPI-2 expression is

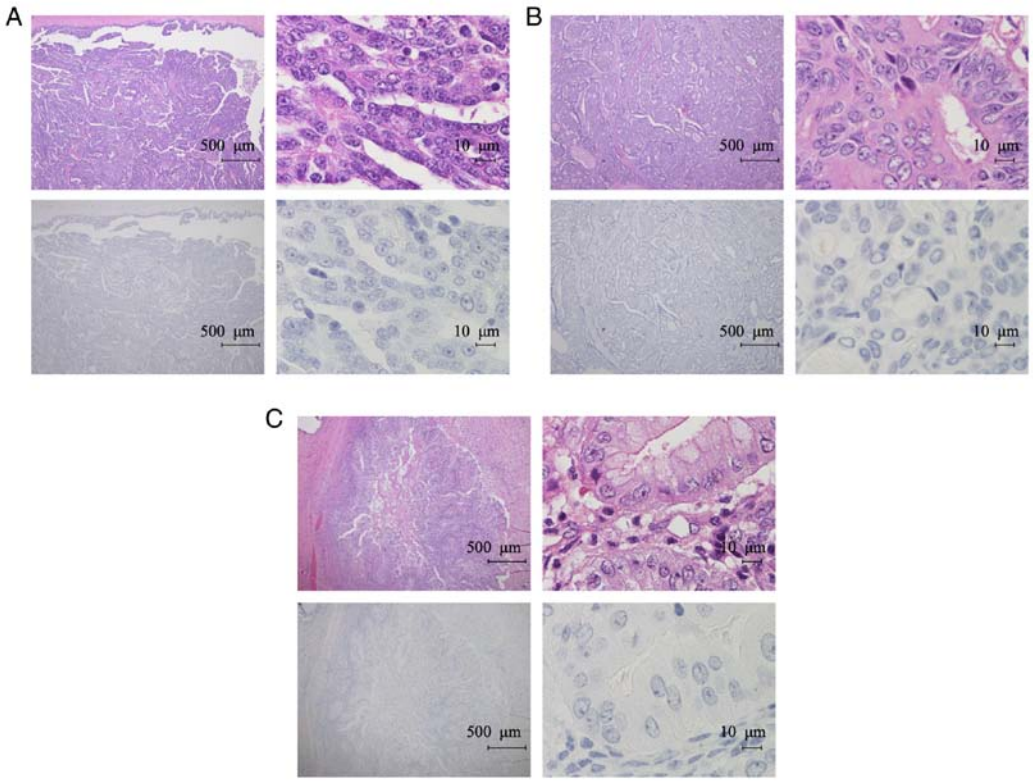

Figure 4. IHC analysis of TFPI-2 in non-CCC EOC tissues. Representative images of H&E staining (top) and IHC for TFPI-2 (bottom) in non-CCC tissues. (A) High grade serous carcinoma. (B) Endometrioid carcinoma. (C) Mucinous carcinoma. IHC, immunohistochemistry; TFPI-2, tissue factor pathway inhibitor-2; CCC, clear cell carcinoma; EOC, epithelial ovarian cancer; H&E, hematoxylin and eosin.

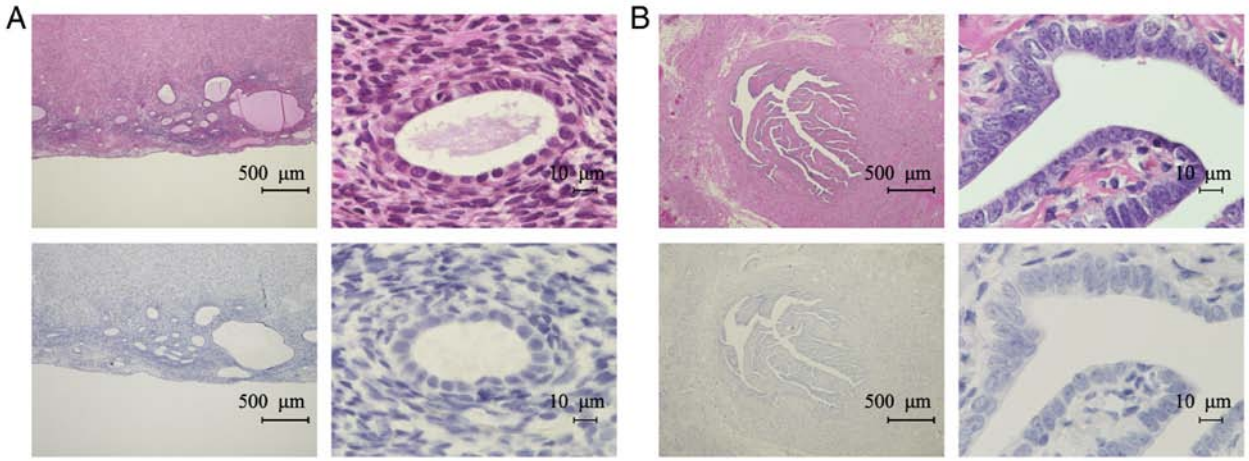

Figure 5. IHC analysis of TFPI-2 in non-neoplastic tissues. Representative images of H&E staining (top) and IHC for TFPI-2 (bottom) in non-neoplastic tissues. (A) Endometrium of TFPI-2-negative case. (B) Fallopian tube epithelium. IHC, immunohistochemistry; TFPI-2, tissue factor pathway inhibitor-2; H&E, hematoxylin and eosin.

epigenetically silenced by aberrant methylation of CpG islands in the TFPI-2 promoter (16,20). In contrast, our study showed that TFPI-2 is elevated in the serum of OCCC patients and is certainly expressed in OCCC tumor cells. These findings suggest that the roles of TFPI-2 may vary depending on the cancer type and that the function of TFPI-2 in ovarian CCC is unique compared with its role in other cancers. In this study, we excluded cases that received neoadjuvant therapies to precisely evaluate the TFPI-2 expression dynamics in OCCC tissues, and therefore the enrolled patients were predicted to have an inherent good prognosis and likely to be in early stages. This bias could be another possibility to explain the negative correlation of TFPI-2 expression and clinical aggressiveness in OCCC tissue. Further studies are needed to elucidate the potential value of TFPI-2 as a prognostic marker or monitoring marker for OCCC patients.

In conclusion, we confirmed the expression of TFPI-2 in clinical OCCC tissues and confirmed the nuclear, cytoplasm, and ECF/ECM subcellular localization of TFPI-2 in cultivated OCCC cells and surgical tissues. We also demonstrated the

high specificity of TFPI-2 expression in OCCC tissues. TFPI-2 expression in IHC may support its use for diagnosis of OCCC in combination with existing markers.

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Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request. Aperio's annotation software is available at https://www.leicabiosystems.com/digital-pathology/Accessed 13/07/2010.

Authors' contributions

YO contributed to the methodology, software, formal analysis, investigation, and writing of the original draft. SK contributed to the methodology, writing of the review and editing. YN contributed to the investigation. MY contributed to the investigation. TT contributed to the investigation. SS contributed to the investigation. SM contributed to the investigation. NO contributed to the investigation. HK contributed the resources and conducted the data curation. TY conducted the validation and contributed to the resources. EM was responsible for the conceptualization and supervision. YM contributed to the conceptualization, validation and writing of the review and editing as well as the supervision. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The experimental protocol of the present study was reviewed and approved by the Institutional Review Board of Kanagawa Cancer Center Hospital (approval no. ethics-2018-10). Written informed consent was obtained from the patients for publication of the study and accompanying images.

Patient consent for publication

Not applicable.

Competing interests

SM and NO are employees of the Tosoh Corporation, which is now developing an *in vitro* diagnosis approach for ovarian CCC patients by evaluating blood TFPI-2 concentration. EM obtained a grant from Tosoh Corporation, outside the submitted work. YM obtained grants from Tosoh Corporation, both for this work and outside the submitted work. The other authors have no conflicts of interest directly relevant to the content of this article.

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