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Tissue factor pathway inhibitor 2 (TFPI2) is a potential serum biomarker for clear cell renal carcinoma

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Renal and ovarian clear cell carcinoma (CCC) are both characterized by a clear cytoplasm and exhibit similar genomic alterations and clinical characteristics. We hypothesized that both CCCs may share clinical biomarker. Tissue factor pathway inhibitor 2 (TFPI2), a serine protease inhibitor, has emerged as a promising serum biomarker for ovarian CCC, and we evaluated the efficacy of TFPI2 as a biomarker for renal cell carcinoma (RCC). Serum samples were collected from patients with RCC and healthy volunteers, and TFPI2 levels were measured. Expression of *TFPI2* in each cell type was evaluated using single-cell RNA sequencing. Survival analyses according to *TFPI2* expression levels were performed based on publicly available databases. Serum TFPI2 was significantly elevated in patients with RCC compared to healthy volunteers, particularly those with clear cell histology. Metastatic RCC tumors exhibited higher TFPI2 than localized RCCs. Moreover, higher TFPI2 correlated with higher Fuhrman grades in clear cell RCC. Publicly available databases showed an association between *TFPI2* expression and overall survival, particularly in clear cell RCC. Single-cell RNA sequencing confirmed *TFPI2* expression in clear cell RCC and normal kidney tubular epithelial cells. TFPI2 has emerged as a potential serum biomarker for RCC, offering avenues for improved detection and prognostication.

Keywords Serine protease inhibitor, Placental protein, Biomarker, Kidney cancer, Clear cell carcinoma

Worldwide, renal cell carcinoma (RCC) is the sixth and tenth most commonly diagnosed cancer in men and women, accounting for 5% and 3% of all tumors, respectively[1]. This prevalence has been fueled by an increase in tumors incidentally diagnosed on imaging studies such as ultrasonography and computed tomography. A critical issue in RCC is that no specific serum biomarker has yet been established for its detection.

The predominant pathological subtype of RCC is clear cell carcinoma (CCC), which accounts for approximately 75–80% of RCC tumors[2,3]. CCC is also found in ovarian malignancies, with an incidence of 10–27% of all epithelial ovarian cancers[4,5]. Renal and ovarian CCCs are both characterized by a clear cytoplasm due to the accumulation of cytoplasmic glycogen and genomic studies have shown several mutational similarities between these two diseases, including frequent alterations in the chromatin remodeling and the mammalian target of rapamycin pathway[6]. Renal and ovarian CCCs also exhibit similar clinical characteristics, such as resistance to standard chemotherapies[7]. Therefore, we hypothesized that renal and ovarian CCCs may share clinical biomarkers.

A serine protease inhibitor, tissue factor pathway inhibitor 2 (TFPI2; also known as placental protein 5)[8], has been identified as a highly specific serum biomarker for predicting ovarian CCC[8–10]. A modified proteomics technique, "secretome," was used to identify TFPI2 in media conditioned by CCC-derived cell lines[8]. Our previous study developed a highly efficient automated enzyme-linked immunosorbent assay for TFPI2 detection and determined an adequate cutoff level of serum TFPI2 to differentiate between patients with CCC and those with other epithelial ovarian cancers and borderline tumors or benign ovarian lesions, including endometriosis[9]. Based on our hypothesis that renal and ovarian CCCs may be able to share a clinical biomarker, we validated the

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performance of TFPI2 as a specific serum biomarker for the preoperative prediction of renal CCC in a single-center study.

Material and methods

Patients

TFPI2 levels were measured in patients with RCC and healthy volunteers at a single-center (Yokohama City University Hospital). All patients with RCC underwent radical nephrectomy between 2001 and 2023 for suspected renal masses and were diagnosed with renal malignancies based on pathological findings. Healthy voluntary samples were collected anonymously by the Tosoh Corporation (Tokyo, Japan) or the Biobank at Yokohama City University. This study was performed in accordance with the Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research Involving Human Subjects, after approval by the Institutional Ethics Committee of Yokohama City University (B181100031, B200800009, and B210300038).

Measurement of serum TFPI2 values in patients with RCC and healthy volunteers

Preoperative serum samples were collected in Venoject II serum separator tubes (VPAS109K60, Terumo, Tokyo, Japan) and stored for the analysis of TFPI2 serum concentration. The tubes were stored for 2–3 h at 4 °C or 30 min at room temperature, and then centrifuged at 1000–1500 *g* for 10 min. Serum aliquots were stored between − 40 and 80 °C. TFPI2 concentrations in each serum sample were measured at the Department of Clinical Laboratory at Yokohama City University Hospital or Tosoh Corporation using reagents provided by the Tosoh Diagnostics Product Division (Tosoh Corporation, Tokyo, Japan). The measurements were performed by clinical laboratory technologists who were blinded to the sample information. TFPI2 concentration was measured by the direct assay method using an automated immunoassay analyzer system (Tosoh Corporation), as described in our previous study[8].

Expression analysis of TFPI2 in renal tumor tissue and generation of Kaplan–Meier survival curves derived from the publicly available databases

The UCSC Xena database (https://xena.ucsc.edu/) provided the bulk RNA-seq data. For comparison of *TFPI2* gene expression levels in normal tissues and renal CCC tissues, "GDC TCGA Kidney Clear Cell Carcinoma (KIRC)" from the UCSC Xena was used. *TFPI2* expression in normal tissues (72 samples) and primary tumors (534 samples) was compared.

The UCSC Xena data was also used to perform prognostic analysis according to the expression levels of *TFPI2* in TCGA database. "GDC TCGA Kidney Clear Cell Carcinoma (KIRC)", "GDC TCGA Kidney Papillary Cell Carcinoma (KIRP)", and "GDC TCGA Kidney Chromophobe (KICH)" were divided into two groups based on median *TFPI2* expression levels in each dataset including clear cell, papillary, and chromophobe RCCs, respectively.

Identification of RNA expression of TFPI2 using single-cell RNA sequencing

We previously performed single-cell RNA sequencing of twelve surgically resected specimens from seven patients, including one Birt-Hogg-Dubé (BHD)-associated hybrid oncocytic chromophobe tumor (HOCT), one BHD-associated chromophobe RCC, one primary lesion, one lymph node metastasis from hereditary leiomyomatosis and renal cell cancer (HLRCC)-associated kidney cancer, two von Hippel-Lindau (VHL)-associated kidney cancers, one sporadic renal CCC, three intratumoral samples from a second sporadic renal CCC, and two normal kidney tissues[11]. We obtained the single-cell transcriptomes of 108,342 cells from these 12 tissues and divided them into 46,890 immune and 61,452 nonimmune cells using CD45, an immune cell marker. Nonimmune cells were annotated into cell clusters using previously reported marker genes for intercalated or principal cells of the collecting duct, distal tubules, loops of Henle, proximal tubules, glomerulus/vascular, and kidney cancers.

We analyzed this dataset following the methodology used in previous studies[11]. A total of 61,452 nonimmune cells were analyzed using the R package "Seurat" (version 3.1.2)[12]. The FindNeighbors (dims = 1:10) and FindClusters functions (resolution = 0.8) were used as parameters, and default settings were used for all other parameters. Each cluster was annotated based on the expression of existing marker genes as described in previous studies. Finally, the expression of the *TFPI2* in each cluster was visualized using UMAP and violin plots. To validate the accuracy of this single-cell analysis, the expression of the *CA9* in each cluster was also visualized.

Statistical analyses

Continuous variables were reported as means and standard deviations. Comparisons between two groups were performed using Student's t, chi-square, or Welch's tests. For multiple comparisons of TFPI2, the Kruskal–Wallis *H* test was used. To compare survival curves, a log-rank test was performed between the two groups. The selection thresholds were as follows: log-rank P value < 0.05 (two-sided) and hazard ratio (HR) within the 95% confidence interval (CI). Spearman's coefficient values were used for the correlation analysis. All statistical analyses were two-sided, and statistical significance was set at $P < 0.05$. All analyses were performed using SPSS software (version 28.0, Armonk, NY, IBM Corp.). Graphs were generated and receiver operating characteristic (ROC) curve analysis was performed using GraphPad Prism software (version 9.0, San Diego, CA, USA).

Results

Background of enrolled patients with RCC and healthy volunteers

The characteristics of patients with localized (N = 42) and metastatic (N = 12) RCC are summarized in Tables 1 and 2, respectively. The average ages at diagnosis were 64.0 ± 10.7 and 64.8 ± 10.9 years in patients with localized

Number of patient			42
Age, yr, median (range)			64 (40–80)
Female/Male, n			9/33
Tumor size, cm, mean ± SD			5.7 ± 2.6
Histology, n (%)	Clear cell		37 (88.1)
	Non-clear	Papillary	2 (4.8)
		Chromophobe	1 (2.4)
		Chromophobe & collecting duct	1 (2.4)
		Neuroendocrine	1 (2.4)
	lyv (+)		22 (52.4)
pT stage, n (%)	1a		13 (31.0)
	1b		7 (16.7)
	2a		3 (7.1)
	2b		1 (2.4)
	3a		11 (26.2)
	3b		4 (9.5)
	4		3 (7.1)

Table 1. Clinicopathologic characteristics of patients with localized renal cell carcinoma.

Number of patient			12
Age, yr, median (range)			65 (40–80)
Female/Male			2/10
Tumor size, cm, mean ± SD			7.6 ± 3.5
Radical nephrectomy/Biopsy	7/5		
Histology, n (%)	Clear cell		9 (75)
	Non-clear	Papillary	2 (16.7)
		Mucinous tubular spindle cell	1 (8.3)
	*Lymph vascular invasion positive		7 (100)
IMDC classification, n (%)	Good	5 (41.7)	
		Intermediate	2 (16.7)
		Poor	5 (41.7)
Metastatic lesions, n (%)	Lymph node		8 (66.7)
	Lung		8 (66.7)
	Bone		5 (41.7)
	Liver		5 (41.7)
	Adrenal gland	1 (8.3)	
	Contralateral Kidney	1 (8.3)	
	Spleen		1 (8.3)
	Pleural		1 (8.3)
	Salivary gland	1 (8.3)	
	Parotid gland	1 (8.3)	

Table 2. Clinicopathologic characteristics of patients with metastatic renal cell carcinoma. *Evaluated in radical nephrectomy cases only.

and metastatic renal carcinoma, respectively. In both groups, the predominant histological finding was CCC (88.1% of localized and 75% of metastatic tissue samples). In the patients with metastatic renal carcinoma, the International Metastatic RCC Database Consortium (IMDC) risk classification was good (41.7%), intermediate (16.7%), or poor (41.7%). The predominant metastatic lesion sites were the lymph nodes and lungs (66.7%).

A total of 241 healthy volunteers, consisting of 139 women and 102 men, were enrolled to serve as controls, and their average age was 43.5 ± 9.7 years. Average serum TFPI2 value in the control group was 130.4 ± 35.4 pg/mL, and there were no significant sex differences between female (131.9 ± 35.2 pg/mL) and male (128.4 ± 35.7 pg/mL) volunteers ($P = 0.440$; Figure S1A). Spearman's coefficient values between TFPI2 concentrations and age were 0.074 ($P = 0.389$; Figure S1B) in female and 0.036 ($P = 0.717$, Figure S1C) in male volunteers.

Serum TFPI2 levels in patients with RCC and healthy volunteers

Serum TFPI2 levels were significantly higher in patients with RCC ($P<0.001$; Fig. 1A), especially in those with CCC ($P<0.001$; Fig. 1B). Metastatic RCC samples showed higher TFPI2 expression than localized RCC ($P<0.001$) and localized RCC levels were higher than those in healthy volunteers ($P=0.042$; Fig. 1C). When focusing only on CCC, Fuhrman grades 3–4 showed higher serum TFPI2 levels than grades 1 ($P=0.042$) and 2 ($P=0.007$; Fig. 2A). The Spearman's coefficient between the tumor size of localized CCC and serum TFPI2 was 0.113 (P = 0.500; Fig. 2B). The cutoff point for TFPI2 to distinguish between patients with localized CCC and healthy controls was 170.0 pg/mL with 59.46% sensitivity and 88.36% specificity based on ROC curve analysis (Fig. 2C).

mRNA expressions of TFPI2 in RCC and normal kidney tissue

The GDC data set showed that mRNA expression of *TFPI2* indicated no significant difference between RCC and normal kidney tissues at the bulk level of mRNA-seq (Fig. 3A). However, the Kaplan–Meier curve derived from UCSC Xena data suggested that higher expression of *TFPI2* was associated with a significantly shorter overall survival (OS) of patients than lower *TFPI2* expression ($P<0.001$; data not shown). With respect to histological types, worse OS with higher expression of *TFPI2* was prominent in CCC ($P<0.001$), but not in papillary (P = 0.258) or chromophobe (P = 0.176; Fig. 3B) tumors.

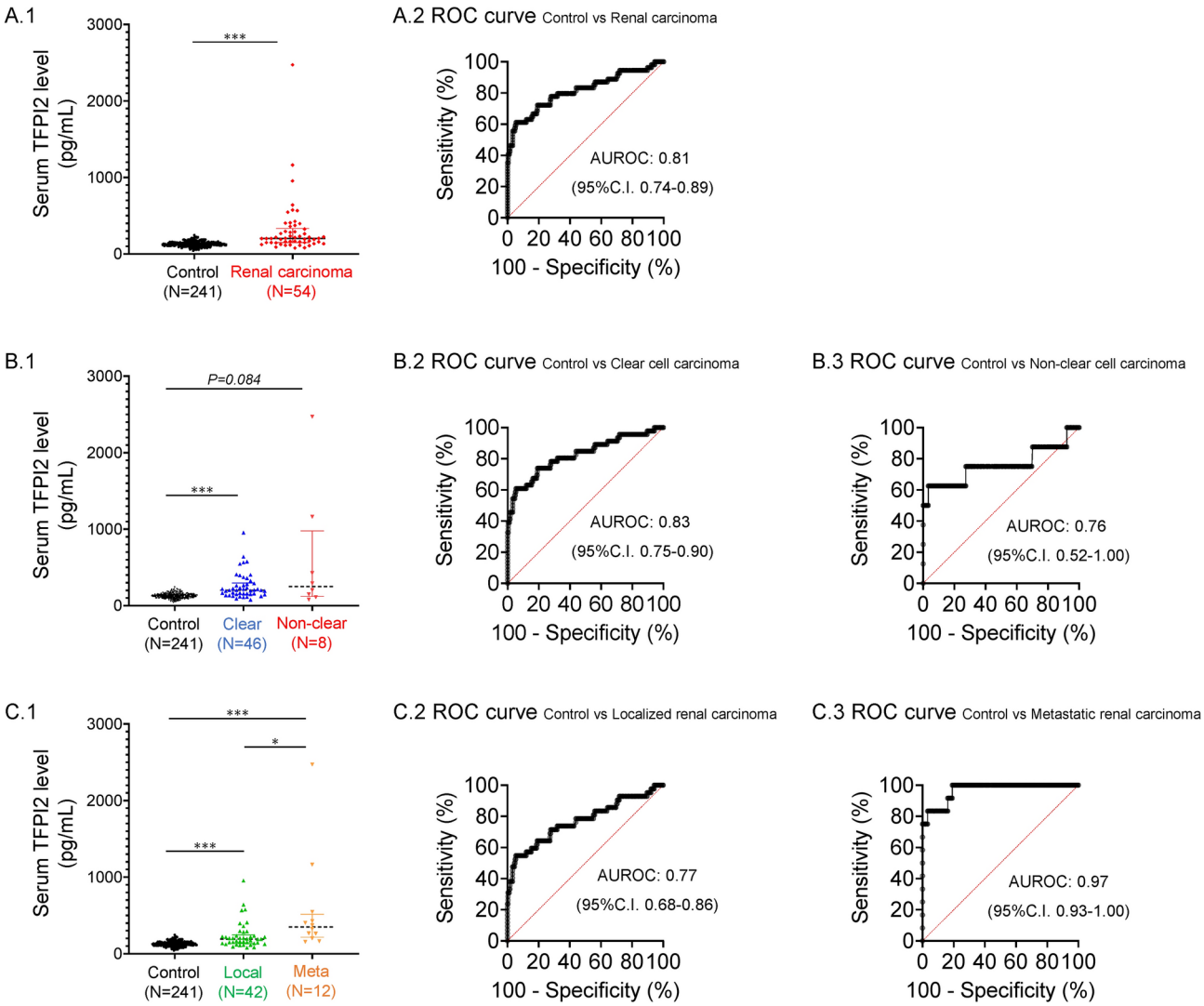

Fig. 1. Dot plot analysis comparing serum TFPI2 levels in control and patients with renal cell carcinoma (RCC) and receiver operating characteristics analysis of TFPI2 between 2 groups (**A**), Dot plot analysis comparing serum TFPI2 concentrations in control and patients with clear and non-clear RCC and receiver operating characteristics analysis of TFPI2 among 3 groups (**B**), and Dot plot analysis comparing serum TFPI2 levels in control and patients with localized or metastatic RCC and receiver operating characteristics analysis of TFPI2 among all 3 groups (**C**).

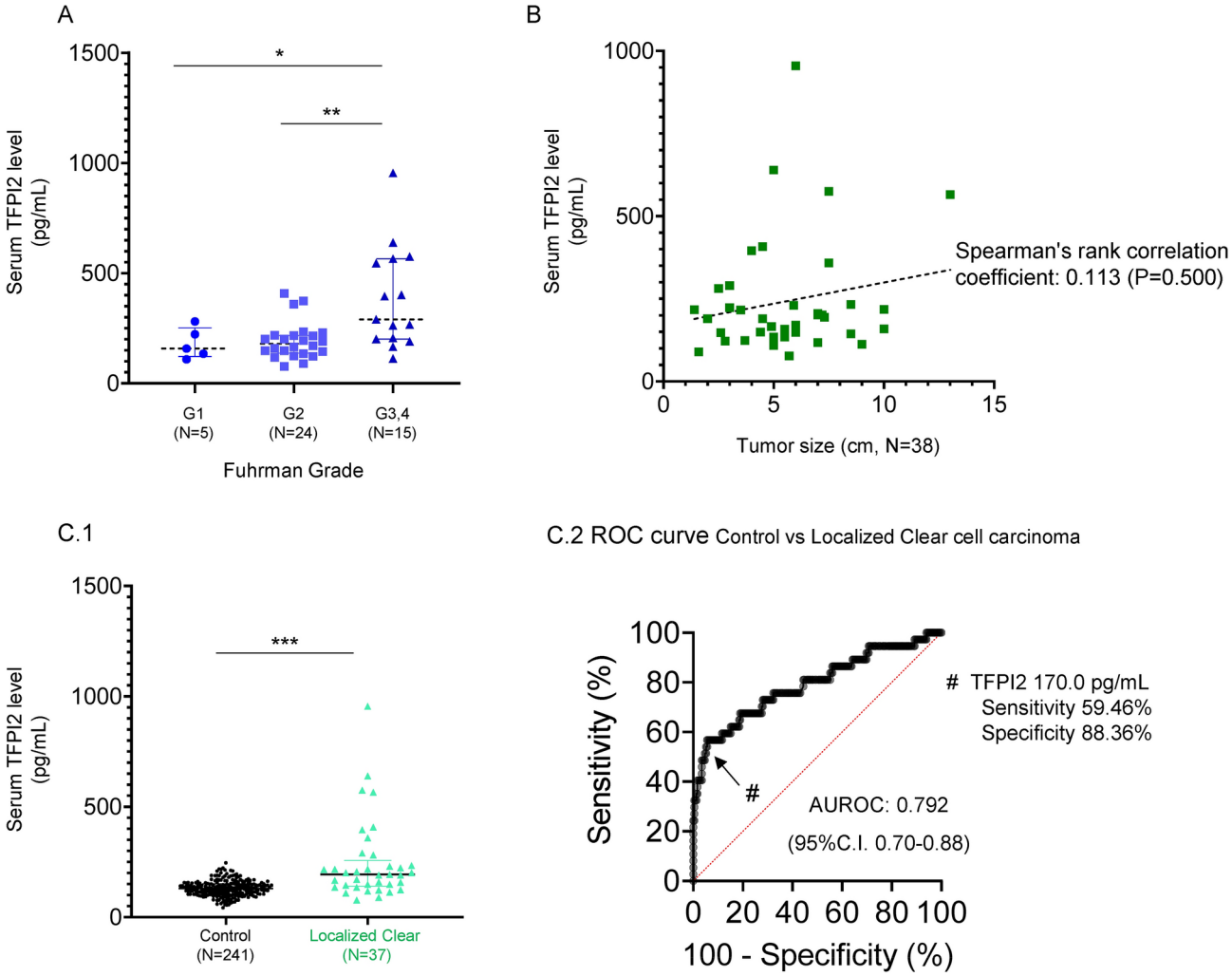

Fig. 2. Serum TFPI2 levels in patients with renal clear cell carcinoma (CCC) according to Fuhrman grade (**A**) and Correlation between serum TFPI2 concentration and renal tumor size in patients with renal CCC (**B**).

RNA expression of TFPI2 in single-cell RNA sequencing

Using single-cell RNA sequencing, the expression of *TFPI2* was identified in CCC using a UMAP plot (Fig. 4A). The expression of *TFPI2* in CCC was significantly higher than that in normal kidneys, including the loop of Henle, distal tubule, and glomerular vasculature as shown in the violin plot ($P < 0.001$; Fig. 4B). *CA9* expression was only confirmed in the CCC cluster (Supplementary Figs. 2A and 2B).

Discussion

The current study demonstrates that the serum TFPI2 is a promising biomarker for renal CCC, a predominant malignant subtype of RCC. In this study, serum TFPI2 could discriminate patients with RCC, especially CCC with 170.0 pg/mL as possible cutoff value and could be a possible prognostic marker based on the analysis of publicly available data which showed that the mRNA expression of *TFPI2* was associated with oncological outcome. Seeking biological evidence for the predictive and prognostic potential of serum TFPI2 in RCC, single-cell transcriptome analysis revealed significantly higher *TFPI2* expression in CCC of RCC than in normal kidney cells.

The most common histological types of renal cell carcinoma are CCC (75–80% of RCC), papillary (10–15%), chromophobe (5%), and other rare forms, such as collecting duct carcinoma (< 1%), comprise the remainder[2,3]. To date, the early detection of RCC has been hindered by the absence of effective serum biomarkers[13,14], although plasma cytokines or circulating proteins have been detected to predict the efficacy of pharmacological treatment for metastatic RCC[15–17]. Indeed, the precise prediction of RCC, especially CCC, within a real-world clinical setup, including serum or urine tests, is a key unmet need.

Tissue factor pathway inhibitor-2 (TFPI-2) is a structural homolog of tissue factor pathway inhibitor (TFPI), an endogenous inhibitor of tissue-factor-dependent blood coagulation[18]. TFPI-2 plays a key role in cancer progression by acting as a serine protease inhibitor that suppresses tumor growth and metastasis through its effects on coagulation, angiogenesis, extracellular matrix degradation, and gene regulation via epigenetic

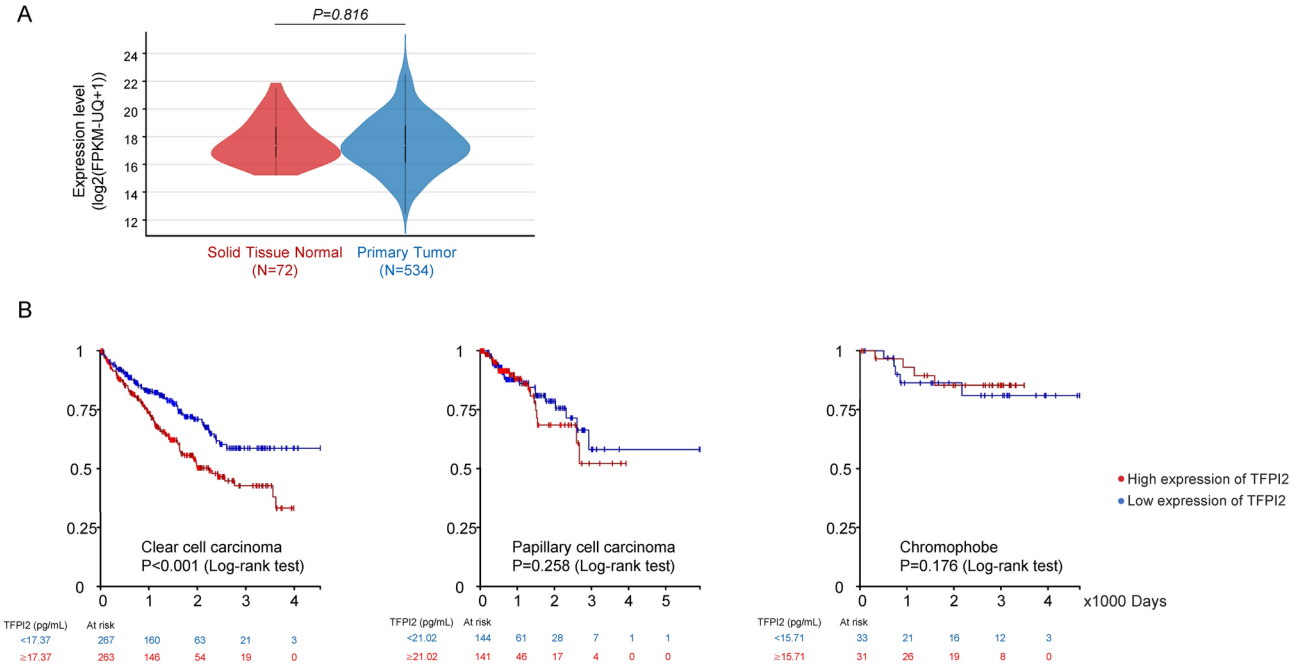

Fig. 3. Comparison of *TFPI2* expression in normal solid kidney tissue and primary renal carcinoma at bulk level of mRNA-seq (**A**) and Kaplan–Meier overall survival probability and *TFPI2* expression levels in three renal cell carcinoma histological subtypes. (1. Clear cell, 2. Papillary and 3. Chromophobe) (**B**).

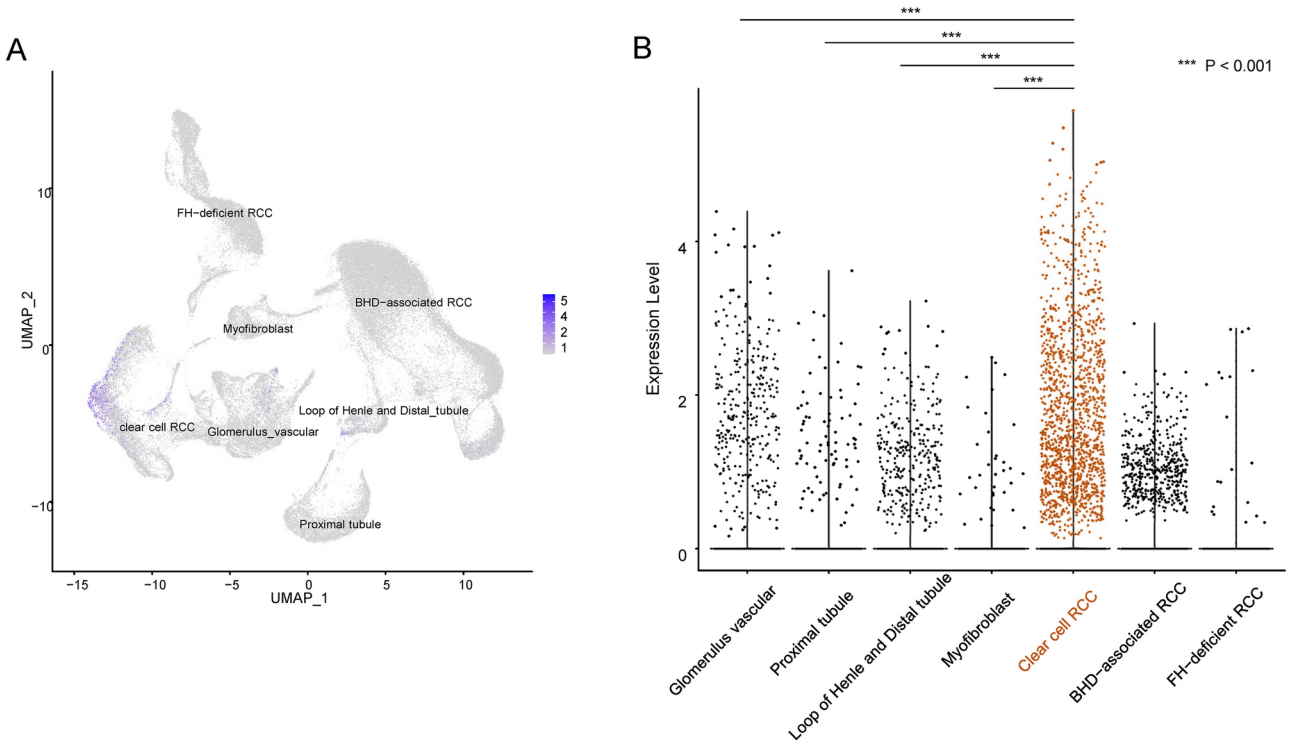

Fig. 4. UMAP plot (**A**) and violin plot (**B**) of the expression of the *TFPI2* gene in single-cell RNA sequencing of renal cell carcinoma and normal kidney tissues.

mechanisms[19]. Reduced expression of TFPI-2 is commonly observed in various cancers, where it correlates with advanced tumor stages, suggesting its potential as a biomarker[20]. Additionally, TFPI-2 promotes cell apoptosis and regulates cell survival through intracellular signaling, with actions both inside and outside of cells that influence the tumor microenvironment and internal cellular pathways[19]. Interestingly, while TFPI-2 expression is diminished in many cancers[19,21], it is elevated in ovarian CCC[22].

This study showed that elevated serum TFPI2 in patients with renal CCC was similar to its previous discovery in ovarian CCC[9]. This finding indicated that renal and ovarian CCC shared not only similar morphological but also biological characteristics. In fact, genomic studies have demonstrated several mutational similarities between renal and ovarian CCC, including frequent alterations in the chromatin remodeling SWI-SNF and cellular proliferation phosphoinositide 3-kinase-mammalian target of rapamycin pathways, as well as a shared hypoxia-like mRNA expression signature[6]. In this study, renal CCC-specific elevation of serum TFPI2 was consistent with the mRNA expression pattern observed in single-cell RNA sequencing. Also, TFPI2 expression was significantly higher in renal CCC tumor cell clusters than in all normal kidney cell clusters at the single-cell level, although there was no difference between normal and tumor cells in the bulk data. These findings suggest that the upregulation of *TFPI2* in tumor tissues is responsible for the high levels of serum TFPI2 in patients with renal CCC.

Furthermore, high TFPI-2 expression in tumor tissue was associated with worse OS in publicly available data (UCSC Xena). Interestingly, the opposite relationship has been observed in other cancers, and hypermethylation of the TFPI-2 gene promoter was higher in metastatic cancers as opposed to localized tumors, and under-expression of TFPI-2 was associated with poor prognosis and metastasis[23–25]. The exact role of TFPI-2 in cancer progression and possible approaches to down- or up-regulate TFPI-2 expression warrant further studies.

Human RCC tumors are thought to arise from a variety of specialized cells located along the length of the nephrons. Both CCC and papillary RCC are thought to arise from the epithelium of the proximal tubules[3]. Chromophobe RCC, oncocytoma, and collecting duct RCC are believed to arise from the distal nephrons, probably from the collecting tubule epithelium. In this study, single-cell transcriptome analysis indicated *TFPI2* expression in the renal tubular epithelium of normal kidneys, supporting the oncological origin theory of TFPI2 expression in CCC.

TFPI2, also known as placental protein 5 (PP5)[26,27], is abundantly produced in the placenta and significantly elevated in the serum of pregnant women[28]. However, this study found no difference in serum TFPI2 values between female and male volunteers. In addition, this study showed no evidence of an effect of age on serum TFPI2 levels in either female or male volunteers. In fact, TFPI2 is now utilized to predict ovarian CCC in clinics without any correction for patient background[9]. Thus, we believe that TFPI2 could be used as a robust serum biomarker for the simple screening of patients with renal CCC, potentially monitoring tumor recurrence and even predicting OS in daily clinical use.

Although this study provides valuable insights into the potential of TFPI2 as a serum biomarker for renal CCC, this study had several limitations. First, the study was conducted at a single center, which may have limited the generalizability of the findings. Second, the sample size, particularly for certain subgroups such as patients with metastatic RCC, was relatively small, limiting the statistical power and robustness of the results. This study primarily focused on preoperative serum TFPI2 levels and their association with RCC diagnosis. Third, long-term follow-up data, including recurrence rates, disease progression, and OS, was not collected in this study and may provide a more comprehensive assessment of TFPI2's prognostic value in RCC. Finally, TFPI2's diagnostic and prognostic utility should be externally validated in independent cohorts in future studies.

Conclusion

TFPI2 has emerged as a potential serum biomarker for renal CCC, offering avenues for improved detection and prognostication, similar to its utility in ovarian CCC. However, the clinical utility of TFPI2 warrants further exploration in routine diagnostic and monitoring practices for patients with RCC. Single-cell transcriptome analysis further elucidated the *TFPI2* expression patterns, confirming its relevance in renal CCC.

Data availability

The data that support the findings of this study are available on request from the corresponding author, [H.I.].

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Author contributions

Hiroki Ito: Writing – original draft ; methodology ; formal analysis ; writing – review and editing ; Prepared Figs. 1, 2 and Supplementary Figure Ryosuke Jikuya: Writing—original draft ; methodology ; formal analysis ; Prepared Figs. 3 ,4 Shohei Myoba: Formal analysis Takaaki Inoue: Writing – review and editing (supporting) Tomoyuki Tatenuma: Data Curation Go Noguchi: Data Curation Daiki Ueno: Data Curation Yusuke Ito: Data Curation Mitsuru Komeya: Data Curation Kentaro Muraoka: Data Curation Masahiro Yao: Supervision ; writing – review and editing Hisashi Hasumi: Supervision Noboru Nakaigawa: Conceptualization ; writing – review and editing Kazuhide Makiyama: Supervision All authors reviewed the manuscript.

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Competing interests

The authors declare no competing interests.

Ethical approval

This study was performed in accordance with the Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research Involving Human Subjects, after approval by the Institutional Ethics Committee of Yokohama City University (B181100031, B200800009, and B210300038).

Informed consent

Written informed consent was obtained from all patients for their data to be used for research purposes.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-80248-x.

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