Renal Replacement Therapy

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Urinary dipeptidyl peptidase-4 is a useful marker for tubulitis, and it is released from the tubular cells of kidney transplant recipients

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Abstract

Background: Biomarkers are needed to diagnose kidney rejection in transplant recipients. We evaluated whether dipeptidyl peptidase-4 (DPP-4) could serve as a biomarker of rejection.

Methods: We determined DPP-4 concentrations and enzymatic activities in serum and urine, as well as DPP-4 expression in 49 kidney biopsy samples from 28 kidney transplant recipients. This study was approved by the ethical standards of the institutional research committee and comply with Helsinki declaration. All patients provided their informed consent. Donors were not from prisoners and were not paid or coerced.

Results: Serum and urinary DPP-4 activities closely correlated with DPP-4 concentrations, but were suppressed by DPP-4 inhibitors. Urinary DPP-4 concentrations increased with acute T cell-mediated rejection (ATCMR; $p=0.030$) and higher Banff t and i scores ($p<0.001$), and correlated with urinary protein/creatinine ratios ($r=0.450$), and inversely with estimated glomerular filtration rate ($r=-0.604$). The area under the receiver operator characteristics curves for urinary DPP-4 concentrations with either Banff t3 or i3 scores were 0.811 (95% confidence interval: 0.687–0.934). The expression of DPP-4 in renal tubular cells was decreased in patients with ATCMR and higher in those with Banff t, i, ct, ci, ah, and ti scores, but was not associated with interstitial fibrosis/tubular atrophy.

Conclusions: We speculated that ATCMR leads to DPP-4 release from tubular cells into urine, resulting in a decrease in tubular cell expression. If so, then ATCMR would induce the elevation of urinary DPP-4 and could therefore serve as a biomarker of tubulitis.

Keywords: DPP-4, Marker, Kidney transplantation, Tubulitis, Urine, Rejection

Background

Graft survival in kidney transplantation has significantly improved due to advances in immunosuppressive therapy and crossmatch tests. Nevertheless, some recipients lose graft function mostly because of acute T cell-mediated rejection (ATCMR), followed by calcineurin inhibitor nephropathy (CNIN), BK polyomavirus nephropathy (BKVN), or acute tubular necrosis. Kidney allograft function is usually monitored by estimating serum creatinine and urine protein levels. However, 4–50% of clinically asymptomatic recipients have subclinical rejection within one year of transplantation. Since parameters for determining the causes of allograft injury have not been defined [1], additional differential diagnoses are necessary to determine causes of rejection. Diagnosing rejection largely depends on renal graft biopsies that are

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invasive to obtain and lead to bleeding and arteriovenous fistulae. Furthermore, repeat biopsies might deteriorate renal function. Noninvasive diagnostic tools for determining graft dysfunction have been developed, but they are not reliable enough for clinical application [2, 3].

Dipeptidyl peptidase-4 (DPP-4) is one of nine proteins that are highly specific for acute rejection [4]. This 110-kDa glycoprotein is a serine protease that is expressed as a transmembrane ectoenzyme on T-lymphocytes, endothelial cells, and epithelial cells. Soluble DPP-4 is secreted into serum and urine. Its enzymatic domain has peptidase activity that cleaves the N-terminal dipeptide from peptides with proline or alanine at the second position [5]. DPP-4 catabolizes proline-rich peptides and regulates the inactivation of neuropeptides, chemokines, and peptide hormones. Incretin, a peptide hormone that promotes insulin secretion, is inactivated by DPP-4. Therefore, DPP-4 inhibitors are now widely applied to treat diabetes.

The kidney has the most abundant DPP-4 activity [5]. The role of DPP-4 in the kidney and its association with chronic kidney disease and metabolic diseases have been investigated [6]. Inhibiting DPP-4 confers kidney preservative effects in rat models of ischemia–reperfusion [7] and tacrolimus-induced kidney damage [8]. From an immunological perspective, DPP-4 inhibitors prolong graft survival in rat models of heart transplantation [9]. Perfusion with a DPP-4 inhibitor reduces allograft rejection rat models of lung transplantation [10]. The expression of DPP-4 on the surface of infiltrating T cells has also been investigated in models of kidney transplantation [11–13]. However, the biological and diagnostic roles of DPP-4 in blood, urine, and kidney grafts are unclear in human recipients of kidney transplants.

Here, we measured urinary and serum DPP-4 concentrations, assessed DPP-4 expression in kidney allografts, and then analyzed their relationships with allograft and clinical status in kidney transplant recipients.

Materials and methods

Patients and samples

We obtained 49 biopsy samples from transplanted kidneys in 28 patients at Yamagata University Faculty of Medicine, Japan, between July 25, 2017, and February 19, 2019. All the patients had been administered with immunosuppressive tacrolimus, mycophenolate mofetil (MMF), methylprednisolone, and basiliximab. Rituximab was administered to patients at increased immunological risk due to incompatible blood types or positive anti-donor-specific HLA antibodies. Recipients with high antibody titers underwent 2–4 sessions of double-filtration plasmapheresis.

The initial tacrolimus dose was 0.1 mg/kg/day, adjusted to trough levels of 8–10 ng/mL, and then, the target trough level gradually was decreased to 3–5 ng/mL by 12 months after transplantation. The initial MMF dose was 2000 mg/day and then decreased to a posttransplant dose of 1000 mg/day by 3 months. Doses were adjusted according to blood drug concentrations and clinical status.

Biopsies were obtained per protocol at 3 and 12 months after transplantation, and per episode (such as elevated serum creatinine or proteinuria) as required. One, four, and ten patients had four, three, and two biopsies, respectively. Spot urine and serum samples were collected after biopsies. Histological diagnosis was performed by a pathologist according to the Banff 2007 criteria as tubulitis (t), interstitial inflammation (i), tubular atrophy (ct), interstitial fibrosis (ci), arteriolar hyalinosis (ah), or total inflammation (ti). Other clinical data regarding sex, age, hypertension, hyperlipidemia, hyperuricemia, diabetes, serum creatinine, estimated glomerular filtration rates (eGFR), urinary protein/creatinine, and blood concentrations of tacrolimus and MMF were collected retrospectively. All procedures involving human participants were in accordance with the ethical standards of the institutional research committee (IRB approval number: H29-213) and with the 1964 Helsinki declaration and its later amendments. All patients provided their informed consent. Donors were not from prisoners and were not paid or coerced.

Immunohistochemical staining for DPP-4

Tissue sections (3 μM thick) were rehydrated and analyzed using immunohistochemistry (IHC). In brief, kidney sections were deparaffinized in xylene (3 × 5 min), followed by 100% ethanol (4 × 5 min), and then, antigens were retrieved by incubation at 120 °C with tris–EDTA (pH 9) for 20 min. Endogenous peroxidase activity was blocked by immersing the sections in 3.0% H_2O_2 in methanol for 15 min. The sections were then incubated overnight at 4 °C with a rabbit polyclonal anti-DPP-4 antibody (40134; Proteintech Group Inc., Rosemont, IL, USA) diluted 50:1, followed by an amino acid polymer with peroxidase and a secondary antibody reduced to its Fab' fragment (Histofine® Simple Stain MAX PO (MULTI); Nichirei, Tokyo, Japan), at 20 °C for 30 min. The sections were stained with 0.05% 3,3-diaminobenzidine containing 0.01% H_2O_2 at room temperature for 10 min and then counterstained with Mayer hematoxylin. The expression of DPP-4 was evaluated based on the rate of tubules expressing high levels of DPP-4. Glomerular capillaries served as the endogenous positive control, and staining representing tubular DPP-4 expression was scored as 1 (essentially none), 2, 3, and 4 (weaker,

equivalent, and more intense staining, respectively, than glomerular capillaries; Fig. 1). Class 3 and 4 tubules were merged into a group with high expression. The same investigator counted tubules in five random microscopy fields. The DPP-4 positive ratio was determined as the number of tubules with high DPP-4 expression per total number of tubules.

Determination of serum and urinary DPP-4 concentrations

Serum and urinary concentrations of DPP-4 were determined using ELISA kits (K4801-100; BioVision Inc., Milpitas, CA, USA) as described by the manufacturer, and then, samples were stored at − 80 °C. Wells in microtiter plates were coated with anti-DPP-4 antibodies and then incubated with serum and urine samples. Biotin-conjugated anti-DPP-4 antibodies were added, free antibodies were removed by washing the plates, and then, the plates were incubated with streptavidin–horseradish peroxidase (HRP). After X washes with Y buffer, substrate tetramethylbenzidine was added to the wells, and then, DPP-4 concentrations were estimated by colorimetry. Sulfuric acid was added to stop the enzymatic reaction, and DPP-4 concentrations were measured as pg/mL in cuvettes at 450 nm.

Measurements of serum and urinary DPP-4 activity

Serum and urine samples stored at − 80 °C were thawed, and then, DPP-4 enzymatic activities were measured using P-nitroaniline (pNA) as the substrate and kits (BML-AK498; Enzo Life Science, Farmingdale, NY, USA) as described by the manufacturer. The samples were placed in a microplate, and the chromogenic substrate (H-Gly-Pro-pNA) was added; the mixture was incubated at 37 °C and allowed to react. The absorbance at 405 nm increased over time due to substrate decomposition by DPP-4. Therefore, we measured absorbance every minute for 30 min using a microplate reader and plotted it. The slope of relative fluorescence per second (RFU/s) was defined as "DPP-4 activity." The controls contained a fixed amount of substrate and synthetic DPP-4, and the ratios of activity of the control to samples were defined as "DPP-4 activity ratio (%)." We investigated whether serum and urinary DPP-4 activity is inactivated by a DPP-4 inhibitor administered to treat diabetes by comparing activities between two and three patients prescribed with and without DPP-4 inhibitors, respectively. Serum or urine samples were mixed with synthetic DPP-4 and substrate, and then, DPP-4 activities were compared with that in controls containing only synthetic DPP-4 and substrate. All results are shown as averages of duplicate

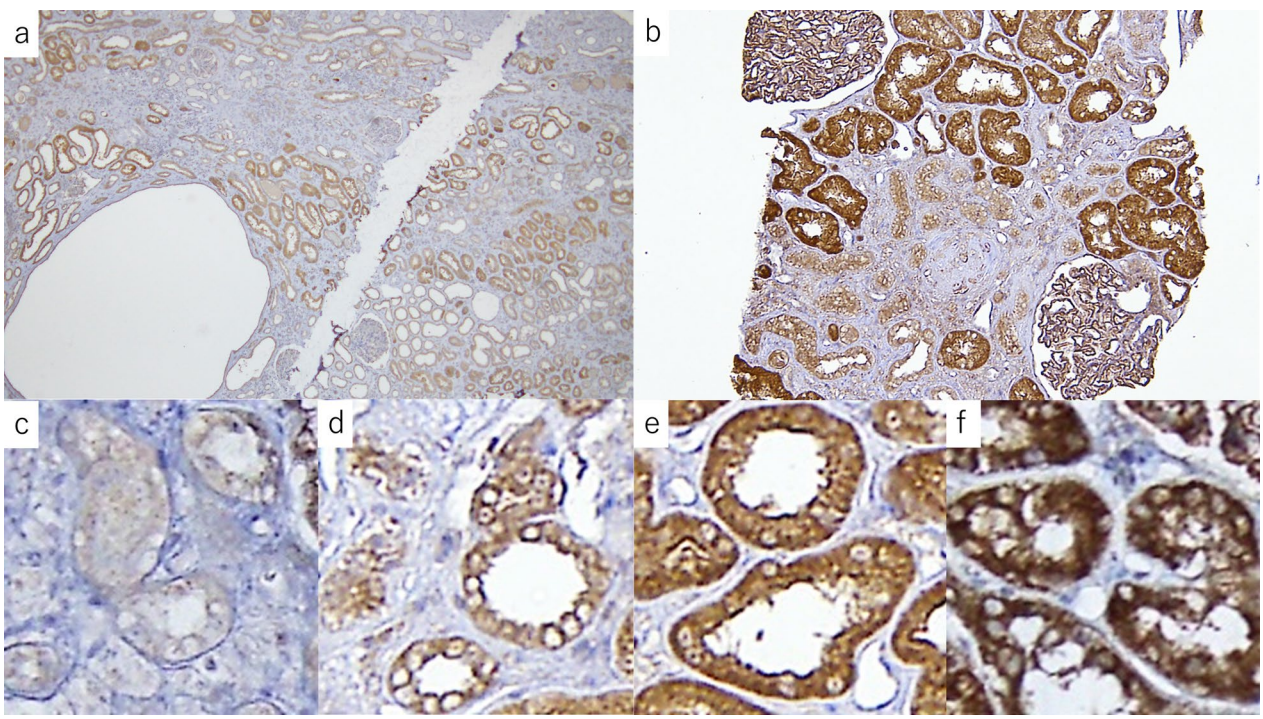

Fig. 1 DPP-4 immunohistochemistry. **a** Immunostaining renal tissue with anti-DPP-4-specific antibody (control). **b** Immunostaining renal biopsy tissue with anti-DPP-4-specific antibody. Glomerular capillary and tubular cells are stained positive (×100). Staining intensity (**c**) 1, essentially none, **d** 2, weaker than that of glomerular capillary, **e** 3, equivalent to that of glomerular loop, **f** 4, more intense than that of glomerular capillary

wells. Only the initial 32 cases were measured because we concluded that the DPP-4 concentration was more useful for the analysis than the DPP-4 activity in those cases.

Statistical analyses

Data were analyzed statistically using EZR© [14]. Between-group differences were analyzed using Mann–Whitney U tests and Kruskal–Wallis tests with Bonferroni adjustment. Correlations between two continuous variables were determined using Spearman rank correlation coefficients. Trends of ordinal variables (Banff scores) were analyzed using Jonckheere–Terpstra tests. Receiver operator characteristics (ROC) curves of sensitivity versus 1-specificity were generated to determine whether urinary DPP-4 can discriminate patients with and without tubulitis. Significant differences were considered at $p<0.05$.

Results

Patients' background

Eleven patients were diagnosed with ATCMR (the most common cause of allograft injury among the patients), and 24 had other clinical abnormalities, such as elevated serum creatinine before biopsy (Table 1).

Serum and urinary DPP-4 concentrations

Urinary DPP-4 enzymatic activity was significantly decreased in patients with diabetes who were treated with DPP-4 inhibitors than those who were not ($p=0.049$). Serum DPP-4 activity also decreased, but the difference did not reach significance ($p=0.073$; Fig. 2a, b). On the other hand, serum and urinary DPP-4 concentrations did not significantly differ between patients with and without DPP-4 inhibitor ($p=0.628$, $p=0.712$, respectively; Fig. 2c, d).

Serum and urinary DPP-4 activities closely correlated with their concentrations in patients without DPP-4 inhibitor ($r=0.784$, $r=0.984$, respectively; Fig. 3a, b). To confirm that the differences were due to the DPP-4 inhibitors, we measured DPP-4 activity in mixtures of synthetic DPP-4 and serum or urine samples from five randomly selected patients, including two who were administered with DPP-4 inhibitors. The activities of DPP-4 in serum and urine in patients with DPP-4 inhibitors were, respectively, lower than control, whereas those in patients without DPP-4 inhibitors were higher (Fig. 3c, d). This implied that the DPP-4 inhibitor circulated in serum and was excreted in urine and that it blocked DPP-4 activity. Thus, the inhibitor affected serum and urine DPP-4 activities, but not concentrations. Therefore, we further investigated relationships between DPP-4 concentrations and the pathological findings of the biopsy specimens.

Table 1 Clinicopathological characteristics of patients

Characteristics	*n*
Total	49
Sex	
Male/female	32/17
Blood type	
Compatible/incompatible	38/11
Donor-specific anti-HLA Ab	
Positive/negative	10/39
Rituximab	
Yes/no	18/31
Donor type	
Living/deceased	46/3
Reason for biopsy	
Protocol/episode	25/24
Number of biopsies	
1	13
2	10
3	4
4	1
Pathological diagnosis	
Normal	29
Acute T cell-mediated rejection	11
Antibody-mediated rejection	1
Interstitial fibrosis/tubular atrophy	3
BK virus nephropathy	3
Calcineurin inhibitor nephropathy	1
Thrombotic microangiopathy	1
Diabetes	
Yes/no	14/35
DPP-4 inhibitor	
Yes/no	4/10
Immunosuppressives	
Tac + MMF + MP	33
CyA + MZ + MP	4
CyA + MMF + MP	3
Tac + MMF + MP + EVL	2
Tac + MZ + MP	2
Tac + MMF	2
Tac + EVL + MP	1
CyA + MP	1
Tac + EVL	1
Median age	48 (20–73) y
Median elapsed time after transplantation	11 (0–52) m

CyA cyclosporine, *EVL* everolimus, *m* months, *MMF* mycophenolate mofetil, *MP* methylprednisolone, *MZ* mizoribine, *Tac* tacrolimus, *y* years

Relationship between serum and urinary DPP-4 concentration levels and pathological diagnosis

Median urinary DPP-4 concentrations were significantly higher in patients with ATCMR than in pathologically normal patients (41.0 vs. 7.36 ng/mL; $p=0.03$; Fig. 4a). The DPP-4 concentrations correlated with high urine protein/creatinine ratios ($r=0.450$), low eGFR ($r=-0.604$), and higher Banff t and i scores (both

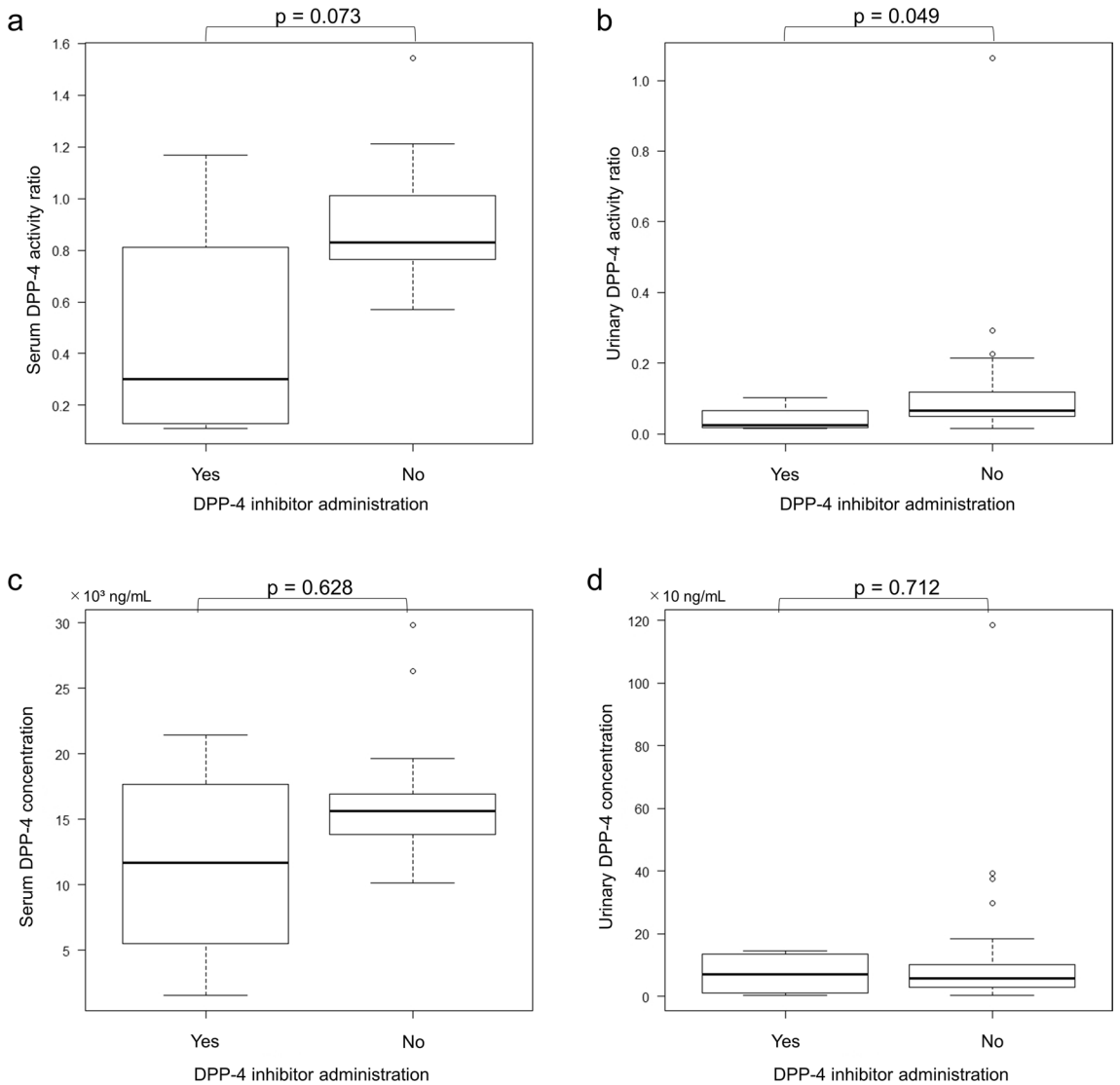

Fig. 2 Activity and concentration of DPP-4 in serum and urine samples with or without DPP-4 inhibitors. Comparisons between **a** serum and **b** urinary DPP-4 activity ratios with and without DPP-4 inhibitor. Comparisons between **c** serum and **d** urinary DPP-4 concentrations with and without DPP-4 inhibitor

$p < 0.001$; Fig. 4b–e). However, urinary DPP-4 concentrations, urinary DPP-4 concentration per urinary creatine concentration, and serum DPP-4 concentrations did not correlate with any other clinical factors.

The area under the ROC curve (AUC) of urinary DPP-4 concentration with Banff scores t3 or i3 was 0.811 (Fig. 5; 95% confidence interval: 0.687–0.934). The cutoff with the maximum Youden index was 48.0 ng/mL (sensitivity, 87.5%; specificity, 60.6%; and negative and positive predictive values 90.9% and 51.9%, respectively).

Relationship between DPP-4 expression and histopathology and pathological diagnosis

Normal tubular cells expressed abundant DPP-4 (Fig. 1d, e). In contrast, expression was decreased in atrophic or inflamed tubular cells (Fig. 1b, c). The median DPP-4 positive ratio was significantly decreased in patients with ATCMR than in pathologically normal patients (50.3% vs. 79.4%; $p < 0.001$; Fig. 6a). The positive ratios decreased as Banff t and i scores increased ($p = 0.043$, $p = 0.009$, respectively; Fig. 6b, c). The ratios also decreased with increasing Banff ci, ct, ah, and ti scores ($p < 0.001$, $p < 0.001$, $p = 0.017$, $p < 0.001$, respectively; Fig. 6d–g), but did not correlate

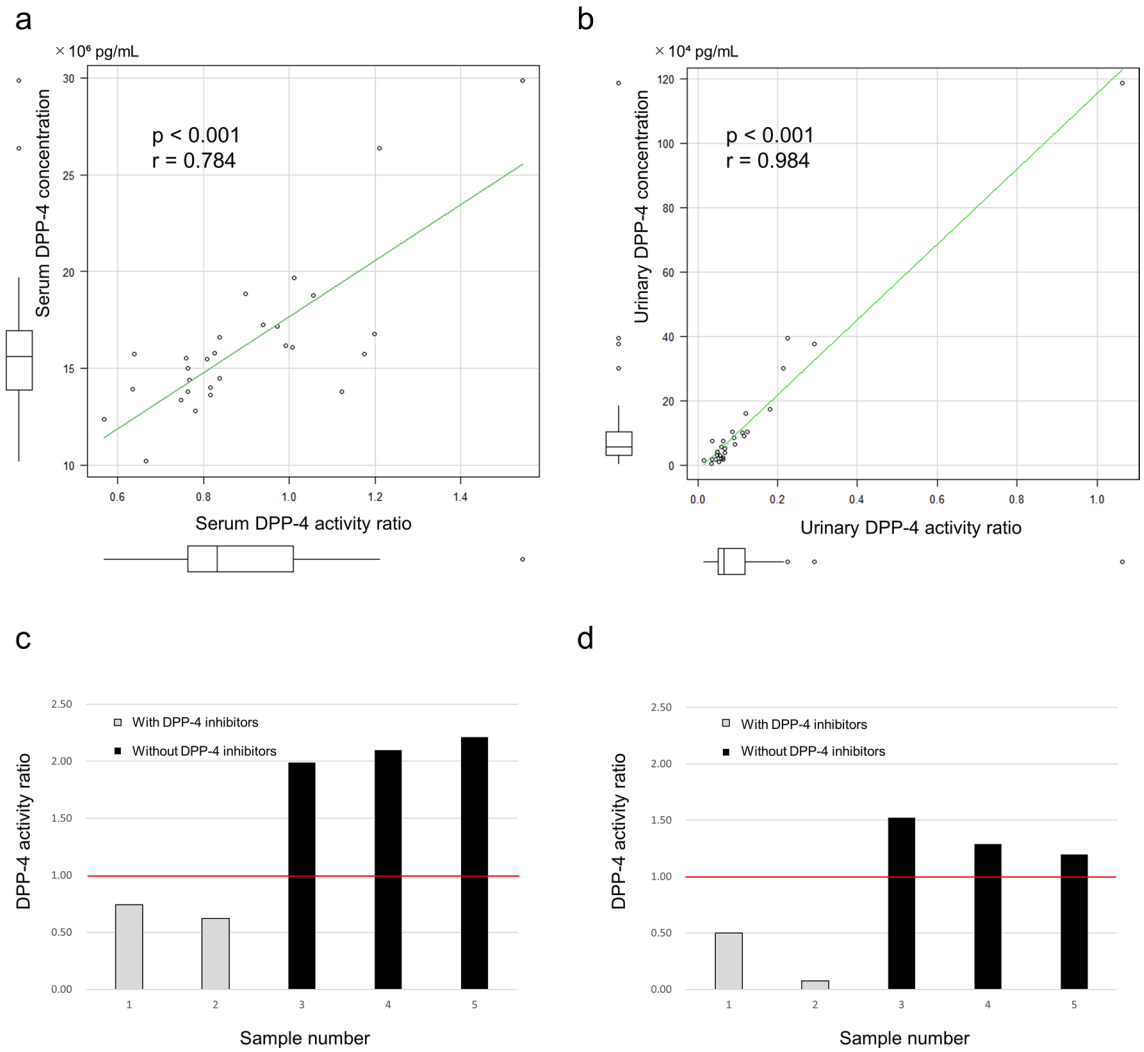

Fig. 3 Relationship between DPP-4 activity and concentrations and DPP-4 inhibitor-induced suppression of DPP-4 activity. **a** Relationship between serum DPP-4 concentration and serum DPP-4 activity ratio. **b** Relationship between urinary DPP-4 concentration and urinary DPP-4 activity ratio. DPP-4 activity ratio when synthetic DPP-4 and **c** serum and **d** urine samples were reacted with substrate. Samples 1 and 2 with, and 3–5, without DPP-4 inhibitors

with clinical interstitial fibrosis/tubular atrophy (IF/TA; $p=0.321$; Fig. 6a).

Discussion

Among the many potential urinary biomarkers that can be applied to diagnose rejection in recipients of kidney transplants, CXCL9 and CXCL10 have very high predictive values for T cell-mediated rejection [15], but highly variable accuracy. These markers are elevated even in BKVN and in patients with antibody-mediated rejection and thus are not specific to ATCMR. Thus, a suitably reliable clinical marker remains to be established [2, 3].

Although tubulitis can be associated with DPP-4 concentrations and enzymatic activity, two studies have indicated that these two parameters do not correlate [16, 17]. However, the present study found otherwise. We also found that DPP-4 inhibitors inhibit serum and urinary DPP-4 activities, but not concentrations. Furthermore DPP-4 inhibitors are applied to treat diabetes mellitus in recipients of renal transplants [18, 19]. Therefore, the DPP-4 concentration is a more effective parameter than DPP-4 enzymatic activity.

We examined correlations between urinary and serum DPP-4 concentrations and tubular DPP-4 expression in

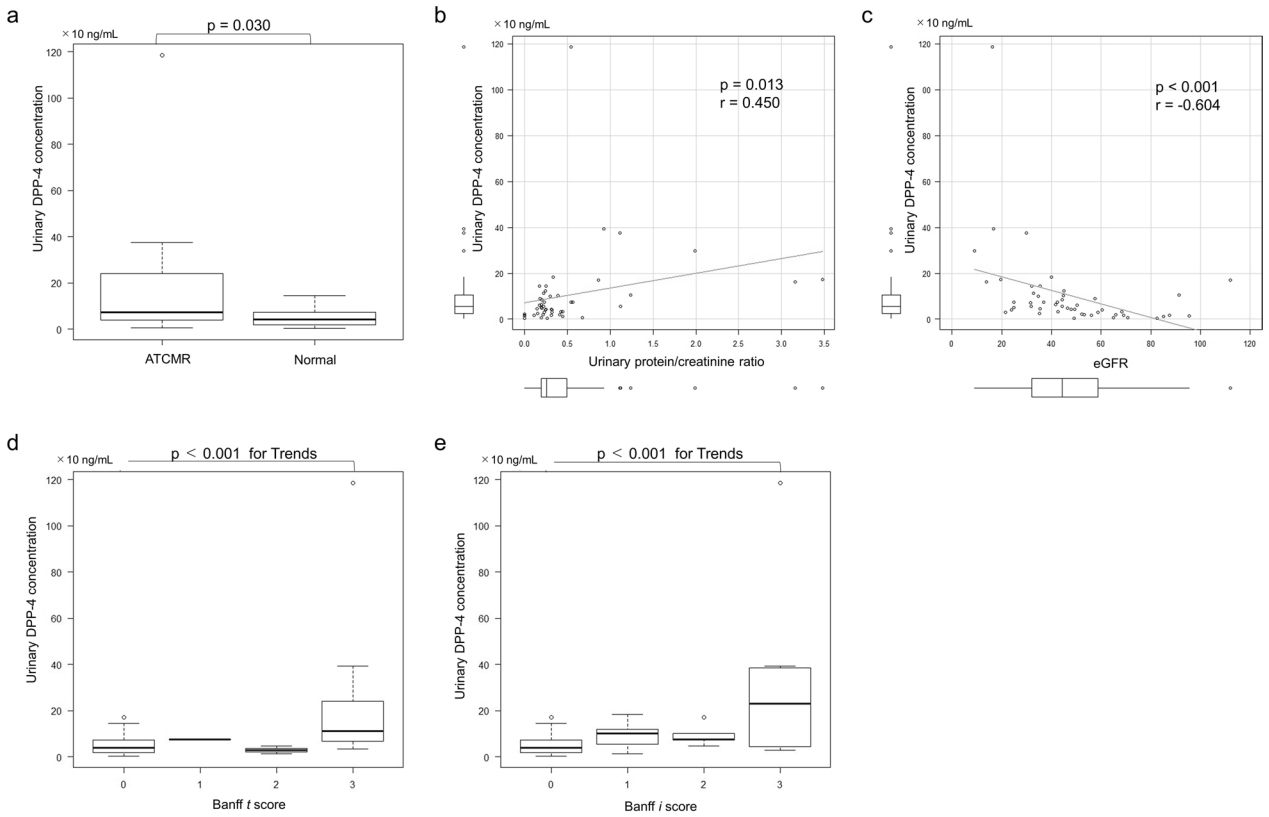

Fig. 4 Relationships between urinary DPP-4 concentration and pathological diagnosis. **a** Comparisons between urinary DPP-4 levels in ATCMR and normal patients. Relationship between urinary DPP-4 concentrations and **b** urinary protein/creatinine ratio and **c** eGFR. Relationships between urinary DPP-4 concentration and **d** Banff t and **e** Banff i scores. ATCMR, acute T cell-mediated rejection; eGFR, estimated glomerular filtration rate

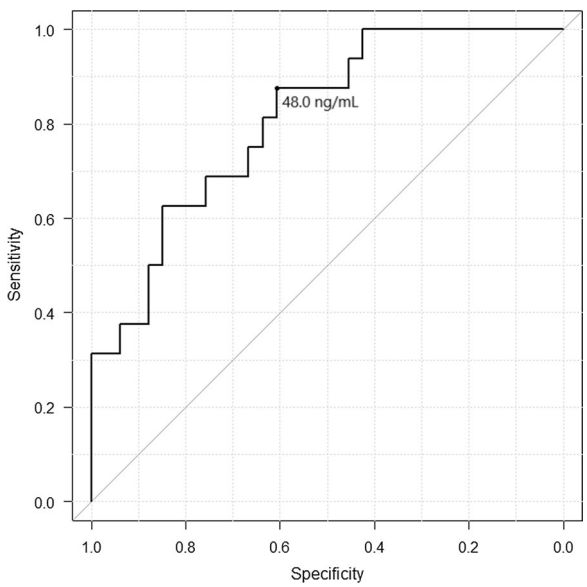

Fig. 5 Receiver operator characteristics curve of urinary DPP-4 concentrations from patients with Banff scores of either t3 or i3

various pathological states. The concentration of urinary DPP-4 closely correlated with ATCMR, which agrees with previous findings [4], as well as with Banff t and i scores, and the AUC was high enough to consider it as a marker of pathological tubulitis.

In contrast, serum DPP-4 concentrations did not correlate with any factors associated with graft status. We suspect that this is because tubular cells in damaged kidneys release DPP-4 into urine, whereas serum DPP-4 is derived from T-lymphocytes in bone marrow [20, 21]. Because DPP-4 expression on T-lymphocytes reflects immunosuppressive status, it might be a marker for determining graft rejection or immune monitoring [12, 13, 22]. Currently, immunosuppression is well controlled by therapeutic drug monitoring, and general immunosuppressive status might not significantly differ among recipients. Here, we confirmed that serum DPP-4 cannot be a marker of rejection or graft status.

The expression DPP-4 in tubular cells showed a strong inverse correlation with Banff ct, ci, and ti scores compared with t and i scores. These scores not only affected

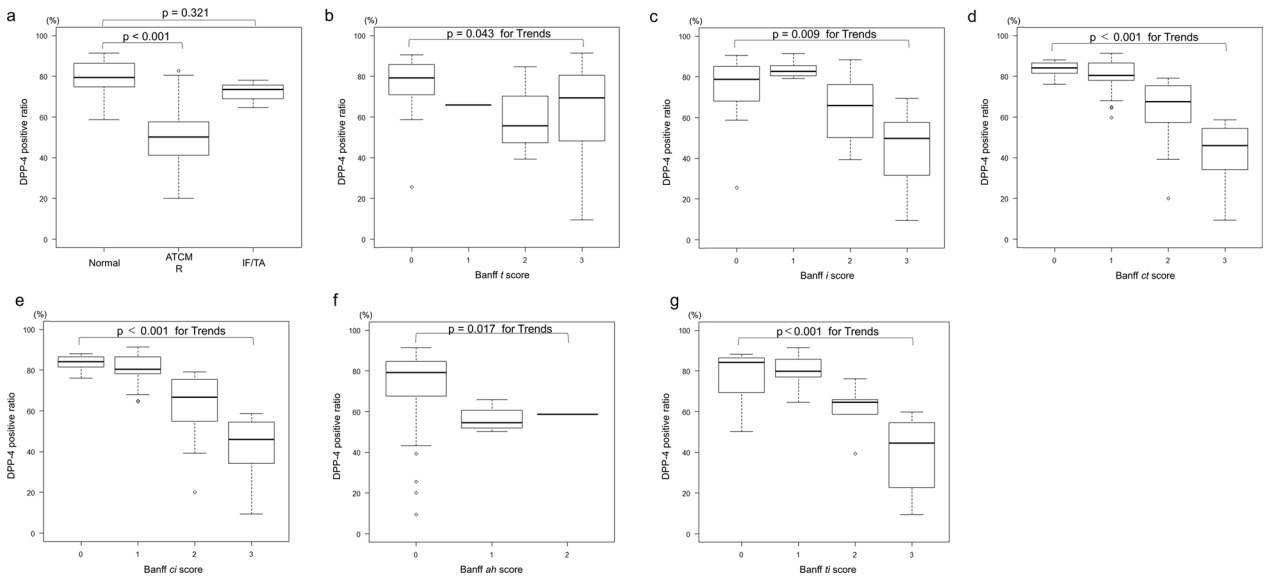

Fig. 6 Relationships between DPP-4 expression assessed by immunohistochemical staining and histopathology. **a** Comparison of DPP-4 positive ratios among control, ATCMR, and IF/TA groups. **b** Relationships between DPP-4 positive ratio and **c** Banff, **d** t, **e** ct, **f** ci, and **g** ti scores. *ATCMR* acute T cell-mediated rejection, *IF/TA* interstitial fibrosis/tubular atrophy

the IF/TA grade, but they were also elevated during rejection and in patients with BKVN and CNIN. Because tubular DPP-4 expression was associated with ATCMR and not IF/TA, we concluded that the elevated ct, ci, and ti scores mainly resulted from ATCMR.

Urinary DPP-4 concentrations correlated with tubulitis, whereas urinary DPP-4 expression in tubular cells inversely correlated with interstitial fibrosis rather than with tubulitis. We postulated that tubulitis initially causes tubular cells to release DPP-4 into urine, and then, prolonged or progressive inflammation leads to interstitial fibrosis, tubular atrophy, and decreased DPP-4 expression. The origin of urinary DPP-4 in recipients of kidney transplants has not yet been proven; in fact, immune cells infiltrating the kidney might be a source of urinary DPP-4. Kasprzycka et al. found no difference in DPP-4 expression between T cells that infiltrated kidney stroma and T cells in peripheral blood [11]. Immune cells infiltrating the kidney are unlikely to affect urinary, more than serum DPP-4. Moreover, urinary DPP-4 is elevated in patients with diabetic kidney disease and tubular injury [23, 24]. Thus, serum DPP-4 cannot be the origin of urine DPP-4 because the serum DPP-4 concentration was not associated with the status of transplanted kidneys and urinary DPP-4 concentrations. Moreover, the urinary concentration of DPP-4 was more closely associated with eGFR than other urinary proteins. Urinary DPP-4 concentrations and DPP-4 expression in renal tubular cells were not apparently related, which might be explained by the time lag between the expression of renal tubular DPP-4 and changes in that of urinary DPP-4. However, further studies are required because this notion has not yet been supported by empirical data.

This study has the following limitations. The small sample size prevented us from analyzing associations between DPP-4 and pathological states such as antibody-related rejection, BKVN, and CNIN. We were also unable to assess correlations between DPP-4 and other tubular markers. We analyzed gene and protein expression only by IHC because we used residual routine biopsy specimens. Finally, we could not verify histological changes induced by DPP-4 inhibitors.

In conclusion, urinary DPP-4 concentrations were increased in patients with ATCMR, and DPP-4 expression in renal tubular cells was decreased in association with fibrosis in such patients. The release of DPP-4 from tubular cells into urine might be induced by ATCMR and result in decreased tubular DPP-4 expression. To our knowledge, this is the first investigation into relationships among urinary, serum, and tissue expression of DPP-4, and its clinical importance in patients with kidney transplants.

Abbreviations

DPP-4: Dipeptidyl peptidase-4; ATCMR: Acute T cell-mediated rejection; CNIN: Calcineurin inhibitor nephropathy; BKVN: BK virus nephropathy; MMF: Mycophenolate mofetil; eGFR: Estimated glomerular filtration rate; ROC: Receiver operator characteristic; IF/TA: Interstitial fibrosis/tubular atrophy.

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Author contributions
AY contributed to all processes of this manuscript and is the corresponding author. HI participated in data analysis and interpretation, especially technical aspects. HN contributed to the study design and kidney transplants. HF contributed to data collection and statistical analysis. NT contributed to the concept, design, and supervision of the study and gave the final approval of the version to be published. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All procedures involving human participants were in accordance with the ethical standards of the institutional research committee (IRB approval number: H29-213) and with the 1964 Helsinki declaration and its later amendments. All patients provided their informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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