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ORIGINAL ARTICLE

Interstitial fibroblasts in donor kidneys predict late posttransplant anemia

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ABSTRACT

Background. Posttransplant anemia (PTA) is associated with the progression of kidney disease and mortality in kidney transplant recipients. Although the main causes of PTA are recipient factors, donor factors have not been fully investigated. In this study we investigated the association of donor pathological findings with the incidence of PTA in kidney transplant recipients after 3 years of transplantation.

Methods. We conducted a retrospective cohort study at a single university hospital. A total of 50 consecutive adult recipients and donors were enrolled. To assess the structure of interstitial lesions, immunohistochemical staining of interstitial fibrosis and fibroblasts were assessed in 0-h biopsies for quantitative analysis.

Results. The incidence of PTA in this cohort was 30%. The mean hemoglobin (Hb) was 11.6 ± 0.8 g/dL in patients with PTA and 14.3 ± 1.5 g/dL in patients without PTA. An inverse association was observed in biopsies between interstitial fibrosis area and interstitial fibroblast area ($P < 0.01$) and each pathological finding was examined for its association with PTA incidence after multivariate adjustment. For the interstitial fibrosis area, the odds ratio (OR) was 1.94 [95% confidence interval (CI) 1.26–2.99; $P < 0.01$]. For the interstitial fibroblast area, the OR was 0.01 (95% CI 0.00–0.16; $P < 0.01$). Receiver operating characteristics curve analysis indicated that the interstitial fibroblast area had high predictive power for the incidence of PTA.

Conclusions. The presence of interstitial fibroblasts in donor kidneys may play an important role in predicting the incidence of PTA.

Keywords: anemia, fibroblast, interstitial fibrosis, kidney biopsy, kidney transplantation

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INTRODUCTION

Anemia is associated with progression of kidney disease or mortality in both chronic kidney disease patients and renal transplant patients [1]. In general, anemia in renal transplant patients is called posttransplant anemia (PTA). The timing of PTA has not been defined uniformly after transplantation, but based on its timing and causes, PTA is generally categorized into two types: early PTA and late PTA. Early PTA can occur up to 6 months after transplantation and affects 70–80% of transplant patients [2–5]. Early PTA has been attributed to various causes, including blood loss at the time of surgery, frequent blood draws, iron depletion [6], erythropoietin (EPO) deficiency, relative resistance to EPO [3, 7], dilution caused by water retention, the persistent effect of uremic toxins [7] and the use of high-dose immunosuppressive agents [4, 5]. In contrast, late PTA usually occurs >2-years after transplantation [4]. Late PTA is sometimes thought to be uncommon during the late posttransplant period, but it actually occurs in 30–40% of transplant patients. Late PTA has also been attributed to various factors, including age (recipient and donor), gender, iron depletion, rejection, chronic inflammation, infection, malignancy and the use of drugs such as angiotensin-converting enzyme inhibitors (ACEIs), angiotensin II receptor blockers (ARBs), immunosuppressive agents, antibiotics and antiviral agents [4, 5].

Although the main causes of PTA are thought to be patient factors, the effects of donor factors have not been fully investigated. The authors of one cohort study found that interstitial fibrosis in donor kidneys predicts PTA [8]. They semiquantitatively evaluated the severity of interstitial fibrosis using 0-h biopsy specimens and reported an association with the incidence of PTA at 1 year posttransplantation. Their results suggested that interstitial fibrosis might cause a loss of EPO production via the transformation of interstitial fibroblasts to myofibroblasts [9, 10]. In previous reports, interstitial fibroblasts were found to be the top candidate among the EPO-producing cells for PTA in a mouse model, including tubular epithelial cells, glomerular mesangial cells and interstitial fibroblasts [9, 10]. However, associations between late PTA and pathological findings have not been evaluated previously in humans.

Based on previous reports, we hypothesized that the percentage area of interstitial fibrosis may be inversely related to that of interstitial fibroblasts in donor kidneys and that these pathological findings may be associated with the incidence of late PTA. Therefore, in this study we examined the association between donors' pathological findings and late PTA using quantitative methods in a retrospective analysis of a renal transplant cohort.

MATERIALS AND METHODS

Patients

We conducted a retrospective cohort study of patients treated with renal transplantation at a single university hospital from 29 October 2003 to 30 January 2014. A total of 62 consecutive adult patients who received primary renal transplantation from a living-related donor were initially eligible for the study. Key exclusion criteria included patients receiving transplantation from living unrelated donors, nonadequate iron status [defined as transferrin saturation (TSAT) level ≤20% and serum ferritin level ≤100 ng/mL] [11, 12], hematologic disorder and 0-h biopsy specimens containing less than six glomeruli. In total, 12 patients were excluded, including 2 patients with nonadequate iron status during the pretransplantation period and 10 patients with no biopsy specimens at 0 h posttransplantation. Therefore 50 patients were finally included in the analysis. The study protocol was reviewed and approved by both the ethical committee of The Jikei University School of Medicine (approval #28-003 8246) and the institutional review board of The Jikei University Hospital. All patients provided written informed consent to participate and the study was conducted in full compliance with the principles of the Declaration of Helsinki.

Immunosuppressive regimens

All recipients received triple immunosuppressive agents consisting of methylprednisolone, a calcineurin inhibitor (tacrolimus or cyclosporine) and antimetabolic agents (mycophenolate mofetil or azathioprine), in addition to basiliximab induction. Rituximab and plasmapheresis were added for patients who underwent ABO-incompatible transplantation.

Patient data and follow-up

Clinical data were collected from the electronic clinical database and patients' medical records. The estimated glomerular filtration rates (eGFRs; in mL/min/1.73 m^2) were calculated using the Japanese eGFR equation [13]. Biopsy-proven acute or chronic rejection was diagnosed on kidney biopsy according to the Banff 2013 classification [14].

Outcomes

The primary outcome was the incidence of late PTA at 3 years, defined as hemoglobin (Hb) <13 g/dL in men and <12 g/dL in women according to the World Health Organization criteria and the Kidney Disease: Improving Global Outcome guidelines published in 2012 [15, 16].

Immunohistochemical staining

Fifty renal biopsy specimens at 0 h posttransplantation were prepared and cut into 3-μm-thick slices using a microtome. All renal biopsy specimens contained at least seven glomeruli [17]. To assess the structure of interstitial lesions, two types of immunohistochemical (IHC) staining were performed. First, picrosirius red staining under polarized light, which is specific for collagen types I and III, was performed to identify fibrosis [18–20]. A picrosirius red stain kit was used according to the manufacturer's protocol (Polysciences, Warrington, PA, USA). Second, triple IHC for platelet-derived growth factor receptor (PDGFR)-β, CD34 and α-smooth muscle actin (SMA) was performed to identify fibroblasts that have the ability to produce EPO [10, 21]. Triple IHC was used because single IHC had limited sensitivity and specificity for detecting EPO-producing cells [21]. Using this method, the cytoplasm and nuclei of fibroblasts were positive for blue staining (PDGFR-β) and negative for red (CD34) and brown staining (α-SMA). IHC was performed as described previously [22] and detailed procedures are described in the 'Supplemental methods' section of the Supplementary data.

Morphometric analysis

For the quantitative analysis of fibrosis and fibroblasts in renal interstitial lesions, each picrosirius red– and triple-IHC-stained specimen was measured using the public domain National Institutes of Health software ImageJ [23]. The area of interstitial fibrosis was defined as the ratio of picrosirius red–stained area

Table 1. Recipient characteristics at 3 years posttransplantation

Characteristics	Late PTA group ($n = 15$)	Nonlate PTA group ($n = 35$)	P-value
Hb (g/dL)	11.6 ± 0.8	14.3 ± 1.5	<0.0001
Mean age (years)	36 ± 11	39 ± 10	0.39*
Male, *n* (%)	10 (67)	21 (60)	0.66†
BMI (kg/m^2)	21.5 ± 2.9	22.5 ± 4.0	0.70*
Primary disease, *n* (%)			0.67†
CGN	10 (67)	27 (77)	
DM	1 (7)	3 (9)	
Hypertension	2 (13)	2 (6)	
Other	2 (13)	3 (9)	
Duration of dialysis (months)	11 (10–24)	20 (8–45)	0.19‡
ABO incompatibility, *n* (%)	5 (33)	16 (46)	0.42†
HLA locus mismatch (*n*)	2.5 ± 1.5	2.5 ± 1.4	0.46*
Immunosuppressive drug user, *n* (%)			
Mycophenolate mofetil	15 (100)	31(91)	0.24†
Tacrolimus	15 (100)	32 (91)	0.24†
Cyclosporine	0 (0)	3 (9)	0.24†
Azathioprine	0 (0)	2 (6)	0.35†
Tacrolimus trough level (ng/mL)	4.3 (3.7–5)	4.3 (3.4–5.9)	0.92‡
Fe (μg/dL)	91 (75–119)	74 (61–95)	0.21‡
Ferritin (ng/mL)	61 ± 36	67 ± 46	0.77*
TSAT (%)	31 (28–43)	25 (21–33)	0.29‡
ESA user, *n* (%)	2 (13)	0 (0)	0.027†
Intact parathyroid hormone (pg/mL)	75 ± 29	74 ± 41	0.94*
C-reactive protein (mg/dL)	0.03 (0.03–0.06)	0.03 (0.03–0.10)	0.52‡
ACEI or ARB user, *n* (%)	11 (73)	13 (37)	0.019†
Creatinine (mg/dL)	1.5 (1.0–1.9)	1.2 (1.1–1.5)	0.09‡
eGFR (mL/min/1.73 m^2)	43 ± 12	49 ± 11	0.12*
Acute/chronic rejection rates, *n* (%)	3 (20)	6 (17)	0.81†
Cytomegalovirus (CMV)/BK virus (BKV) infection rates, *n* (%)	7 (47)	10 (29)	0.22†
Incidence rates of malignancies, *n* (%)	0 (0)	0 (0)	–

Values are presented as mean ± SD or median (25th–75th percentiles) unless stated otherwise.
*Unpaired *t*-test.
†Chi-square test.
‡Mann–Whitney U test.
BMI, body mass index; CGN, chronic glomerulonephritis; DM, diabetes mellitus; Fe, iron; CMV, cytomegalovirus; BKV, BK virus.

to the renal cortical area in each whole biopsy specimen, expressed as a percentage. The interstitial fibroblast area was defined as the ratio of the blue-stained area to the tubulointerstitial area in triple-IHC-stained specimens. Ten tubulointerstitial areas were randomly selected from the specimens and evaluated and the mean percentage area of fibroblasts was subsequently calculated. The detailed procedures are described in the 'Supplemental methods' section of the Supplementary data. The researchers who performed all image analyses were blinded to the patients' clinical data.

Statistics

Patient characteristics were compared using the unpaired *t*-test or the Mann–Whitney U test for continuous variables and the chi-square test for categorical variables. In an analysis of the incidence of late PTA, logistic regression was used to calculate each odds ratio (OR) with a 95% confidence interval (CI). Risk factors for late PTA were examined with the use of univariate and multivariate logistic regression. Before multiple regression analysis, multicollinearity was resolved by confirming variance inflation factors. Covariates with $P < 0.05$ in the univariate analysis were included in the multivariate analysis. When covariates reflected the same disease status, a covariate with the lowest P-value in the univariate analysis was selected. The association between interstitial fibrosis and fibroblasts in donor kidneys was evaluated by linear regression. Additionally, receiver operating characteristics (ROC) curves and the area under the curve (AUC) were calculated to compare the predictive powers of interstitial fibrosis and fibroblasts for the incidence of late PTA. All statistical analyses were performed using Stata 14.0 (StataCorp, College Station, TX, USA). A P-value <0.05 was considered to indicate statistical significance for all tests.

RESULTS

Patient characteristics

In total, 50 consecutive renal transplant recipients and donors were analyzed in this study. The incidence of late PTA in this cohort was 30%.

Table 1 summarizes recipient characteristics at 3 years posttransplantation. The recipients' mean Hb level was 11.6 ± 0.8 g/dL in patients with late PTA versus 14.3 ± 1.5 g/dL in those with nonlate PTA ($P < 0.0001$). Patients in the late PTA group were more likely to be treated with an EPO-stimulating agent (ESA) ($P = 0.027$) and ACEIs/ARBs ($P = 0.019$). Apart from recipient drug usage, the characteristics of the other recipients in the late PTA

group did not differ significantly from those of the nonlate PTA group.

Table 2 summarizes donor characteristics at transplantation. The donors' mean age was higher in the late PTA group than in the nonlate PTA group (P = 0.012). Donors with hypertension were more common in the late PTA group (P = 0.006). Other characteristics of donors in the late PTA group did not differ significantly from those of the nonlate PTA group.

Association between late PTA and interstitial fibrosis in donor kidneys

First, the area of interstitial fibrosis was evaluated (Figure 1). The association between the incidence of late PTA and the area of interstitial fibrosis was subsequently analyzed. The area of interstitial fibrosis was larger in the late PTA group than in the nonlate PTA group (P = 0.031) (Figure 2). On multivariate adjustment using five covariates, a significant association was observed between the incidence of late PTA and the area of interstitial fibrosis, independent of other covariates [OR 1.94 (95% CI 1.26–2.99); P < 0.01] (Table 3).

Table 2. Donor characteristics at transplantation

Characteristics	Late PTA group ($n = 15$)	Nonlate PTA group ($n = 35$)	P-value
Mean age (years)	62 ± 7	55 ± 9	0.012*
Male, *n* (%)	4 (27)	10 (29)	0.89†
BMI (kg/m^2)	23.3 ± 2.0	23.3 ± 3.1	0.99*
Hb (g/dL)	13.4 ± 1.2	13.8 ± 1.3	0.32*
DM, *n* (%)	1 (7)	1 (3)	0.53†
HbA1c (%)	5.2 ± 0.4	5.2 ± 0.3	0.94*
Hypertension, *n* (%)	7 (47)	4 (11)	0.006†
Blood pressure (mmHg)			
Systolic	132 ± 17	122 ± 16	0.048*
Diastolic	77 ± 10	72 ± 11	0.14*
Creatinine (mg/dL)	0.6 (0.6–0.7)	0.7 (0.5–0.8)	0.92‡
eGFR (mL/min/1.73 m^2)	73 ± 7	74 ± 11	0.87*
Urine protein excretion (mg/day)	47 (35–102)	46 (20–67)	0.33‡

Values are presented as mean ± SD or median (25th–75th percentiles) unless stated otherwise.
*Unpaired *t*-test.
†Chi-square test.
‡Mann–Whitney U test.
BMI, body mass index; DM, diabetes mellitus.

Association between interstitial fibrosis and fibroblasts in donor kidneys

The interstitial fibroblast area was then evaluated (Figure 3) and its association with the interstitial fibrosis area was analyzed. The interstitial fibrosis area was inversely associated with the interstitial fibroblast area ($r = -0.15$, P < 0.01) (Figure 4).

Association between late PTA and interstitial fibroblasts in donor kidneys

Next, the association between the incidence of late PTA and the interstitial fibroblast area was analyzed. The interstitial

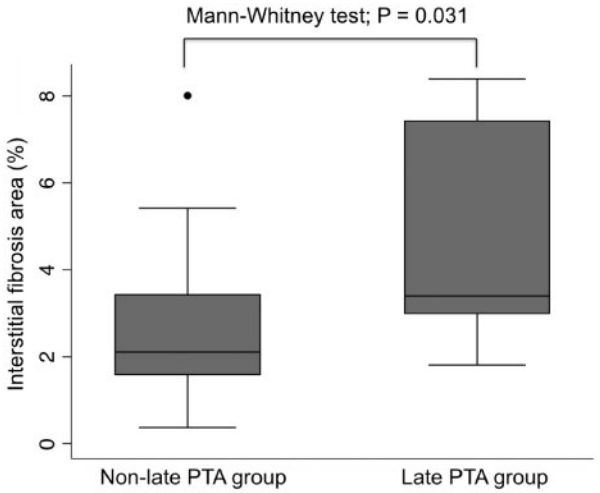

FIGURE 2: Association between late PTA and interstitial fibrosis in donor kidneys. Box plots show the area of interstitial fibrosis between the late PTA group and the nonlate PTA group. Shaded box areas, 25th and 75th percentiles; thick line across each box, median; whisker lines, 95% CI for each category; small circles, outliers.

Table 3. Multiple logistic regression analyses of covariates predicting late PTA

Covariates	OR	P-value	95% CI
Donor with hypertension	10.8	0.014	1.63–71.1
Interstitial fibrosis area at 0-h biopsy	1.94	0.003	1.26–2.99
Recipient ACEI or ARB use	5.67	0.049	1.01–31.9

Multivariate adjustment using the following four covariates: (i) donor age, (ii) donor with hypertension, (iii) interstitial fibrosis area at 0-h biopsy, (iv) ACEI or ARB user at 3 years posttransplant.

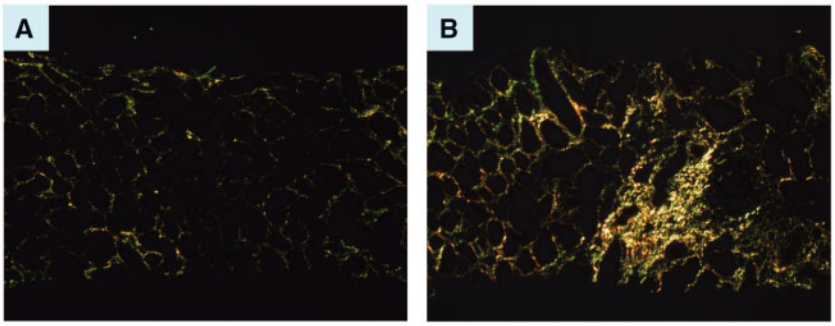

FIGURE 1: Quantitation of interstitial fibrosis using picrosirius red staining under polarized light. Fibrosis is positive for gold staining. Interstitial fibrosis in kidney specimens obtained from recipients with **(A)** nonlate PTA and **(B)** late PTA at 0 h posttransplantation.

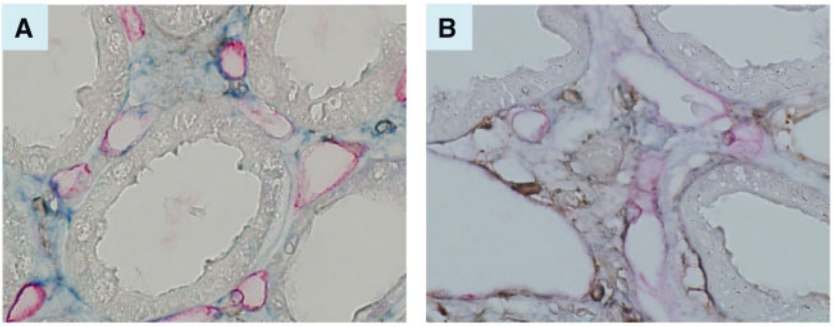

FIGURE 3: Quantitation of interstitial fibroblasts using triple IHC staining. The cytoplasm and nuclei of fibroblasts are positive for blue staining (PDGFR-β) and negative for red (CD34) and brown (α-SMA) staining. Interstitial fibroblasts in kidney specimens obtained from recipients with **(A)** nonlate PTA and **(B)** late PTA at 0 h posttransplantation.

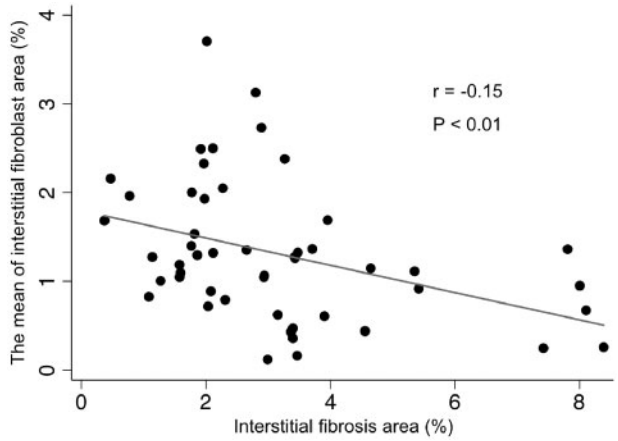

FIGURE 4: Association between interstitial fibrosis and fibroblasts in donor kidneys. Solid circles show recipients at 0-h biopsy. The regression line shows the inverse correlation of the interstitial fibrosis area with the interstitial fibroblast area in donor kidneys.

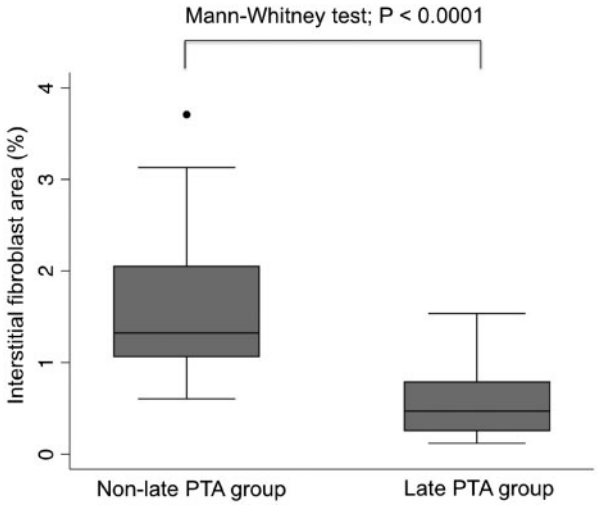

FIGURE 5: Association between late PTA and interstitial fibroblasts in donor kidneys. Box plots show the interstitial fibroblast area between the late PTA and nonlate PTA groups. Shaded box areas, 25th and 75th percentiles; thick line across each box, median; whisker lines, 95% CI for each category; small circles, outliers.

fibroblast area was smaller in the late PTA group than in the nonlate PTA group ($P < 0.0001$) (Figure 5). On multivariate adjustment using four covariates, the association between the incidence of late PTA and the interstitial fibroblast area was reevaluated. A significant association was observed between the incidence of late PTA and the interstitial fibroblast area [OR 0.01 (95% CI 0.00–0.16); $P < 0.01$] (Table 4.).

Comparison of the predictive powers of interstitial fibrosis and fibroblasts for the incidence of late PTA

Finally, ROC curve analysis was performed to estimate the predictive powers of donor pathological findings for the incidence of late PTA (Figure 6). The AUC of the ROC curve for the interstitial fibrosis single model was 0.68, which was subsequently used as a reference model. The AUC of the ROC curve for the interstitial fibroblast single model was 0.90, which was significantly higher than that of the interstitial fibrosis single model ($P = 0.017$). The AUC values of the ROC curve for the interstitial fibrosis/fibroblast dual model was 0.97, which was significantly higher than that of the interstitial fibrosis single model ($P < 0.001$). The AUC of the ROC curve for the interstitial fibroblast single model and interstitial fibrosis/fibroblast dual model did not differ significantly ($P = 0.09$), meaning that the pathological finding of interstitial fibroblasts was a strong single predictive factor.

Table 4. Multiple logistic regression analyses of covariates predicting late PTA

Covariates	OR	P-value	95% CI
Interstitial fibroblast area at 0-h biopsy	0.01	0.001	0.00–0.16

Multivariate adjustment using the following four covariates: (i) donor age, (ii) donor with hypertension, (iii) interstitial fibroblast area at 0-h biopsy, (iv) ACEI or ARB user at 3 years posttransplant.

DISCUSSION

In this study we found significant associations between the incidence of late PTA and the areas of interstitial fibrosis or fibroblasts in donor kidneys, even after adjusting for covariates. We also examined the predictive power of donors' pathological findings in determining the incidence of late PTA using the AUC of the ROC curves. The pathological finding of interstitial fibroblasts may play an important role in predicting the incidence of late PTA.

Late PTA most commonly occurs >2 years after transplantation [4] and affects 30–40% of recipients. The incidence of late

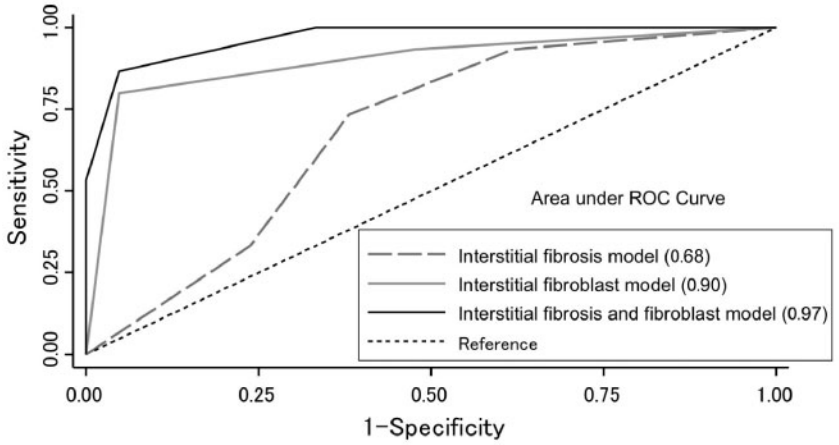

FIGURE 6: AUC of the ROC curve for predicting late PTA. The AUC of interstitial fibrosis model is represented by the dotted gray line, the interstitial fibroblast model is represented by the solid gray line and the interstitial fibrosis and fibroblast model is represented by the solid black line. The dotted black line indicates reference values.

PTA in this cohort was 30%, which is in agreement with previous reports [4].

The authors of a previous cohort study found that interstitial fibrosis in donor kidneys predicts PTA [8]. They evaluated the severity of interstitial fibrosis using 0-h biopsy specimens by semiquantitative methods and reported its association with the incidence of PTA at 1 year posttransplantation. However, the association between PTA, interstitial fibrosis and fibroblasts was not evaluated previously in humans. In this study we found that interstitial fibroblasts may be a stronger factor than interstitial fibrosis in predicting the incidence of late PTA. Interstitial fibrosis is recognized as the final common pathway for PTA, and various factors can contribute to the development of fibrosis. However, interstitial fibroblasts were the top candidates for PTA among the EPO-producing cells, including tubular epithelial cells, glomerular mesangial cells and interstitial fibroblasts in a mouse model [9, 10], which suggests that interstitial fibroblasts may specifically predict the incidence of PTA.

Interstitial fibrosis is known to be associated with renal aging [24, 25] and hypertension [26, 27]. However, we observed no significant associations between aging or hypertension and interstitial fibrosis in this study.

Consistent with previous reports [28, 29], we found that ACEI/ARB users were more common in the late PTA group than in the nonlate PTA group. The mechanism whereby ACEIs/ARBs cause anemia is related to blockade of erythropoietic effects of angiotensin II on red cell precursors as well as improvement in renal blood flow secondary to renal efferent vasodilation, which improves oxygenation [30].

Several limitations of this study should be acknowledged. First, this was a retrospective cohort study with a relatively small cohort size and was conducted in a single center only. Second, we were unable to evaluate EPO deficiency. Although no correlation was observed between serum EPO levels and renal transplant recipients [31, 32], serum EPO levels were not assessed in this study.

Interstitial pathological findings in donor kidneys may be an important factor in predicting the incidence of late PTA. However, associations between interstitial pathological findings and donor outcome have not been fully evaluated. Additionally, a previous study reported tubular engraftment and mesenchymal differentiation of recipient-derived cells after renal transplantation [33, 34]. However, long-term associations between interstitial pathological findings after renal transplantation and the incidence of late PTA have not been evaluated. Thus, further work is required.

CONCLUSION

In conclusion, significant associations were observed between the incidence of late PTA and the areas of interstitial fibrosis or fibroblasts in donor kidneys even after adjusting for covariates. Additionally, interstitial fibroblasts may play an important role in predicting the incidence of late PTA.

SUPPLEMENTARY DATA

Supplementary data are available at ckj online.

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CONFLICT OF INTEREST STATEMENT

None declared.

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