Internal Medicine
The Japanese Society of Internal Medicine
doi: 10.2169/internalmedicine.5455-20
Intern Med 60: 1349-1357, 2021
http://internmed.jp

【 ORIGINAL ARTICLE 】

Anemia in Diabetic Patients Reflects Severe Tubulointerstitial Injury and Aids in Clinically Predicting a Diagnosis of Diabetic Nephropathy

Kenji Ito[1], Soichiro Yokota[1], Maho Watanabe[1], Yori Inoue[1], Koji Takahashi[1], Naoko Himuro[1], Tetsuhiko Yasuno[1], Katsuhisa Miyake[1], Noriko Uesugi[2], Kosuke Masutani[1] and Hitoshi Nakashima[1]

Abstract:

Objective A kidney biopsy is generally performed in diabetic patients to discriminate between diabetic nephropathy (DN) and non-diabetic kidney disease (NDKD) and to provide more specific treatments. This study investigated the impact of anemia on the renal pathology and the clinical course in patients who underwent a kidney biopsy.

Methods We reviewed 81 patients with type 2 diabetes who underwent a percutaneous kidney biopsy. Patients were classified into two groups: isolated DN (DN group, n=30) and NDKD alone or concurrent DN (NDKD group, n=51) groups. The laboratory and pathological findings and clinical courses were investigated.

Results In the NDKD group, membranous nephropathy was the most common finding (23.5%), followed by IgA nephropathy (17.6%) and crescentic glomerulonephritis (13.7%). In the logistic regression analysis, the absence of severe hematuria and presence of anemia were significantly associated with a diagnosis of DN. Akaike's information criterion (AIC) and net reclassification improvement (NRI) analyses revealed improved predictive performance by adding anemia to the conventional factors (AIC 100.152 to 91.844; NRI 27.0%). The tissues of patients in the DN group demonstrated more severe interstitial fibrosis and tubular atrophy (IF/TA) than those in the NDKD group ($p<0.05$) regardless of the rate of global glomerulosclerosis, and IF/TA was related to the prevalence of anemia (odds ratio: 7.31, 95% confidence interval: 2.33-23.00, $p<0.01$) according to a multivariable regression analysis. Furthermore, the isolated DN group demonstrated a poorer prognosis than the NDKD group.

Conclusion DN is associated with anemia because of severe IF/TA regardless of the renal function, and anemia helps clinician discriminate clinically between isolated DN and NDKD.

Key words: interstitial fibrosis, non-diabetic renal diseases, percutaneous kidney biopsy, renal anemia, retinopathy

(Intern Med 60: 1349-1357, 2021)
(DOI: 10.2169/internalmedicine.5455-20)

Introduction

Diabetic nephropathy (DN) is currently a leading cause of end-stage kidney disease (ESKD) worldwide. According to the annual nationwide survey of regular dialysis therapy in Japan, the prevalence of DN among new dialysis patients in 2018 was 42.3% (1), and in this survey, the diagnosis of DN was established clinically in the majority of patients. Generally, a kidney biopsy is considered in diabetic patients who exhibit atypical clinical manifestations as DN, and the main purpose of performing a biopsy is to detect non-diabetic

[1]Division of Nephrology and Rheumatology, Department of Internal Medicine, Faculty of Medicine, Fukuoka University, Japan and [2]Department of Pathology, Faculty of Medicine, Fukuoka University, Japan
Received: May 28, 2020; Accepted: October 14, 2020; Advance Publication by J-STAGE: November 30, 2020
Correspondence to Dr. Kosuke Masutani, kmasutani@fukuoka-u.ac.jp

kidney diseases (NDKD), which can alter the treatment. There have been numerous studies investigating factors discriminating between DN and NDKD, including the duration of diabetes mellitus (DM), diabetic retinopathy (DR), glycemic control, blood pressure, acute kidney injury, and microscopic hematuria, among others (2-6).

Recent studies have suggested the predictive value of anemia in the diagnosis of DN (7, 8). Diabetic patients are known to develop anemia earlier, regardless of the stage of chronic kidney disease (CKD) (9, 10). However, the detailed pathophysiology and significance of anemia in the renal pathology and clinical outcomes are not fully understood.

The present study therefore investigated the impact of anemia on the renal pathology and clinical course in patients who underwent a renal biopsy using a retrospective cohort.

Materials and Methods

Study population

From January 2001 through March 2020, 776 patients underwent a percutaneous kidney biopsy for the diagnosis of primary or secondary kidney diseases at Fukuoka University Hospital. Eighty-one patients (60.4±13.7 years old, 54 men and 27 women) suffering from DM during the biopsy were identified. Written informed consent was obtained for the performance of a percutaneous kidney biopsy and the collection of clinical and pathological data. Blood and renal tissue were sampled only for the diagnosis and clinical management of the patients, and no extra sampling was performed for research purposes.

This retrospective study was performed following the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards and was approved by the Institutional Review Board of Fukuoka University Hospital.

Clinical parameters

The data of urine and blood tests and clinical parameters during the kidney biopsy, including age, sex, duration of DM (defined as a time of hospitalization from the first diagnosis of DM to a kidney biopsy), presence/absence of DR, blood pressure (BP), and the amount of erythropoietin-stimulating agent (ESA) administration, were collected. The clinical outcomes regarding the development of ESKD and death were similarly obtained. The estimated glomerular filtration rate was calculated using the formula of the Japanese Society of Nephrology (11). Anemia was defined as a hemoglobin level <13 g/dL in men and <12 g/dL in women in accordance with the World Health Organization standards. Hypertension was classified as systolic BP >140 mmHg, diastolic BP >90 mmHg, and/or the use of antihypertensive medications (12). Severe hematuria was defined as a urinary red blood cell (u-RBC) count of ≥30/high-power field.

The percutaneous kidney biopsy and pathological interpretation

In all cases, a percutaneous kidney biopsy was performed under ultrasound guidance using 18-gauge needles. Biopsy specimens for light microscopic examination were fixed with 10% neutrally buffered formalin and embedded in paraffin. Sections were cut into 2-μm-thick slices and stained with hematoxylin-eosin, periodic acid-Schiff, periodic acid-methenamine silver, and Masson's trichrome. Frozen sections of biopsy specimens (for direct immunofluorescence staining) were incubated with fluorescein isothiocyanate conjugated antisera to human immunoglobulin (Ig) G, IgA, IgM, Complement (C) 3, C1q, fibrinogen, kappa-light chain, and lambda-light chain. Tissues for the electron microscopic analysis were fixed with 3% glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in a graded alcohol solution, and embedded in Epon 812. Ultrathin sections were cut serially and double-stained with uranyl acetate and lead citrate.

The kidney biopsy specimens were evaluated by two experienced pathologists, and DN was diagnosed based on mesangial expansion or nodular glomerulosclerosis observed under a light microscope. Interstitial fibrosis and tubular atrophy (IF/TA), interstitial inflammation, arteriolar hyalinosis, and arteriosclerosis were also assessed and scored according to the Renal Pathology Society classification for DN (Supplementary material) (13). Glomerular basement membrane (GBM) thickness was directly measured using an electron microscope, and GBM thickening was defined as >430 nm in men and >395 nm in women.

Immunofluorescence assisted in differentiating NDKD from DN. NDKD, such as membranous nephropathy, IgA nephropathy, and minimal change disease, among others, was differentiated by a histopathologic evaluation. Diffuse linear staining of the glomerular and tubular basement membranes for IgG (possibly caused by non-immunological trapping, and non-specific deposition of the IgM, C3, and light chains) was compatible with a diagnosis of DN.

Statistical analyses

All analyses were performed with IBM SPSS version 26 (IBM, Chicago, USA). Data were expressed as the mean± standard deviation or median with interquartile range as appropriate. Differences in continuous variables between the two groups were analyzed by Student's *t*-test or Wilcoxon's rank-sum test, as appropriate. On comparing the three groups, the variables were examined using an analysis of variance or the Kruskal-Wallis test. Consequently, correction for multiple comparisons was performed using Bonferroni's method. The chi-square test or Fisher's exact probability test was used to compare categorical data. Clinical factors associated with the diagnosis of isolated DN and pathological factors related to anemia were identified by univariable and multivariable logistic regression analyses using the forced entry method. Results were expressed as odds ratios (ORs) with respective 95% confidence intervals (CIs).

Table 1. Clinical Characteristics of the Study Population.

	total (n=81)	NDKD (n=51)		isolated DN (n=30)	p value
		NDKD alone (n=37)	NDKD concurrent DN (n=14)		
age (year)	60.4±13.7	61.2±13.2		59.2±14.8	0.529
		62.4±13.4	58.0±12.5		0.494
male [n (%)]	54 (66.7)	36 (70.6)		18 (60.0)	0.329
		27 (73.0)	9 (64.3)		0.523
duration of DM (year)	10 (4-15)	8 (3-13)		13 (8-15) [b]	<0.01
		6 (2-11) [#] [b]	14 (10-23) [#]		<0.01
presence of DR [n (%)]	31 (38.3)	13 (25.5)		18 (60.0) [b]	<0.01
		4 (10.8) [#] [b]	9 (64.3) [#]		<0.01
u-RBC ≥30/HPF [n (%)]	16 (19.8)	14 (27.5)		2 (6.7)	0.023
		10 (27.0)	4 (28.6)		0.124
systolic BP (mmHg)	139.1±21.9	136.8±21.1		144.6±22.4	0.139
		134.5±19.6	141.3±25.5		0.205
diastolic BP (mmHg)	79.3±12.1	80.2±11.7		77.6±12.9	0.358
		78.8±11.6	83.9±11.5		0.275
hypertension [n (%)]	58 (71.6)	34 (66.7)		24 (80.0)	0.199
		22 (59.5)	12 (85.7)		0.078
hemoglobin (g/dL)	12.1 (10.6-14.1)	13.2 (11.35-15.3)		11.2 (10.4-12.5) [b]	<0.01
		13.5 (11.6-15.3) [b]	12.7 (11.1-16.7)		<0.01
anemia [n (%)]	45 (55.6)	21 (41.2)		24 (80.0) [b]	<0.01
		15 (40.5) [b]	6 (42.9)		<0.01
Fe (μg/dL)	71.5±30.4	73.1±29.0		68.9±33.0	0.571
		77.1±27.5	62.8±31.3		0.332
T-sat (%)	31.9 (23.3-36.6)	33.3 (23.6-36.5)		31.4 (23.0-38.0)	0.731
		34.5 (25.7-36.7)	25.3 (18.85-33.8)		0.282
Ferritin (ng/mL)	190.5 (104-396)	185 (104-358)		202 (105-396)	0.605
		199.5 (98.5-307)	172 (111-424)		0.824
albumin (g/dL)	2.65±0.91	2.75±1.01		2.47±0.69	0.144
		2.89±1.11	2.39±0.56		0.084
CRP (mg/dL)	0.16 (0.08-0.4)	0.205 (0.055-0.55)		0.155 (0.10-0.30)	0.918
		0.13 (0.05-0.55)	0.345 (0.10-0.60)		0.382
BUN (mg/dL)	17 (12.5-26.5)	19 (13-27)		15 (12-24)	0.350
		20 (12.5-28)	14 (13-21)		0.374
creatinine (mg/dL)	1.13 (0.80-1.645)	1.13 (0.80-1.68)		1.13 (0.80-1.60)	0.534
		1.115 (0.80-1.935)	1.17 (0.90-1.49)		0.823
eGFR (mL/min/1.73 m^2)	51.91±27.15	50.76±26.45		53.85±28.67	0.624
		50.97±29.12	50.23±18.47		0.884
u-Prot (g/day)	3.5 (1.455-7.3)	3.28 (0.7-5.54)		5.05 (3.2-9.1) [b]	<0.01
		2.95 (0.585-5.485) [b]	3.5 (2.5-5.485)		0.012
nephrotic range proteinuria [n (%)]	44 (54.3)	24 (47.1)		20 (66.7)	0.087
		16 (43.2)	8 (57.1)		0.156
uric acid (mg/dL)	6.20±1.73	6.06±1.62		6.38±1.96	0.472
		6.08±1.64	6.19±1.54		0.752
HbA1c (%)	7.08±1.80	6.87±1.15		7.44±2.55	0.255
		6.69±0.96	7.34±1.49		0.201
TC (mg/dL)	219.4 (179.5-286.2)	220 (176-289.4)		220.2 (198-281)	0.475
		230.5 (187.2-318)	190.4 (172-269)		0.313
TG (mg/dL)	146 (115.5-228.5)	167 (122-230)		143.5 (115-197)	0.348
		172.5 (127.5-225)	141 (114-230)		0.643
HDL-C (mg/dL)	49 (39-68)	49 (38-70)		49.5 (42-64)	0.765
		51.5 (39-73.5)	45 (33-56)		0.148

[#]significant difference between NDKD alone and NDKD concurrent DN (p<0.017, Bonferroni method).

[b]significant difference between NDKD alone and isolated DN (p<0.017, Bonferroni method).

NDKD: non-diabetic kidney disease, DN: diabetic nephropathy, DM: diabetes mellitus, DR: diabetic retinopathy, u-RBC: urinary red blood cell, HPF: high power field, BP: blood pressure, Fe: ferrum, T-sat: transferrin saturation (serum ferrum/total iron binding capacity), CRP: C-reactive protein, BUN: blood urea nitrogen, eGFR: estimated glomerular filltration rate, u-Prot: urinary protein, HbA1c: hemoglobin A1c, HDL-C: high density lipoprotein-cholesterol

Table 2. The Types and Proportions of Clinicopathological Diagnoses Detected across the Cohort.

	NDKD alone (n=37)	NDKD concurrent DN (n=14)	NDKD (n=51)
membranous nephropathy	10 (27.0%)	2 (14.2%)	12 (23.5%)
IgA nephropathy	5 (13.5%)	4 (28.5%)	9 (17.6%)
crescentic glomerulonephritis (pauci-immune)	5* (13.5%)	2** (14.2%)	7 (13.7%)
minimal change disease	6 (16.2%)		6 (11.8%)
obesity related nephropathy	5 (13.5%)	1 (7.1%)	6 (11.8%)
hepatitis virus related nephropathy		4*** (28.5%)	4 (7.8%)
tubulointerstitial nephritis	2 (5.4%)		2 (3.9%)
nephrosclerosis	2 (5.4%)		2 (3.9%)
focal segmental glomerulosclerosis	1 (2.7%)		1 (2.0%)
purpula nephritis	1 (2.7%)		1 (2.0%)
lupus nephritis		1 (7.1%)	1 (2.0%)

*MPO-ANCA positive 4 and ANCA negative 1, **MPO-ANCA positive 1 and proteinase 3 ANCA positive 1, ***hepatitis B virus related 2 and hepatitis C virus related 2.

NDKD: non-diabetic kidney disease, DN: diabetic nephropathy, IgA: immunoglobulin A, MPO: myeloperoxidase, ANCA: antineutrophil cytoplasmic antibody

Differential diagnostic models were developed using the logistic regression model: $\text{logit}(p)=\beta_0+\beta_1X_1+\beta_2X_2+\ldots+\beta_mX_m$ (where X_m is a clinical predictor, β_0 is a constant, and p is the probability of DN diagnosis). A receiver operating characteristic (ROC) curve was illustrated, and the comparison of the area under the curve (AUC), Akaike's information criterion (AIC), and net reclassification improvement (NRI) was conducted to assess the test performance. In the NRI analysis, $p<0.333$, $0.333\leq p<0.667$, and $p\geq0.667$ were considered to indicate low, moderate, and high probability, respectively. Both the renal survival and mortality were assessed using the Kaplan-Meier method followed by the log-rank test. A two-tailed p value less than 0.05 was considered statistically significant.

Results

Clinical characteristics of the study population

The baseline clinical characteristics of each group are shown in Table 1. According to the pathological diagnosis, isolated DN was observed in 30 patients (37.0%) in the DN group, 37 (45.7%) patients in the NDKD alone group, and 14 (17.3%) patients in the NDKD with concurrent DN group. Patients in the NDKD alone and NDKD with concurrent DN groups were combined to form the NDKD group (n =51, 63.0%) for further analyses.

Compared with patients in the NDKD group, those in the DN group showed a long duration of DM, high incidence of DR, low incidence of severe hematuria, low levels of hemoglobin, and high levels of urinary protein excretion ($p<0.05$, using Wilcoxon's rank-sum test, the chi-square test, or Fisher's exact probability test as appropriate). Anemia was present in 45 (55.6%) cases, including 6 [4 (10.8%) in the NDKD group alone and 2 (6.7%) in the isolated DN group] who were treated with ESAs. The rate of patients with anemia was significantly higher in the isolated DN group than NDKD alone group ($p<0.05$, chi-square test). No significant differences were observed in the age, sex, BP, kidney function, or levels of serum albumin, uric acid, hemoglobin A1c (HbA1c), or lipids among the groups.

The types and proportions of clinicopathological diagnoses detected across the cohort are shown in Table 2. In total, there were 11 types of NDKD, including primary and secondary glomerular disease. Of these, membranous nephropathy was the most common (23.5%), followed by IgA nephropathy (17.6%), and crescentic glomerulonephritis (13.7%).

Univariable and multivariable logistic regression analyses in the identification of the factors associated with isolated DN

Clinical variables relevant to the diagnosis of DN were explored (Table 3). In the univariable analysis, nephrotic-range proteinuria and anemia were detected as specific variables ($p<0.1$) along with a long duration of DM, DR, and the absence of severe hematuria, which are well-known conventional factors in the prediction of DN. In a multivariable analysis, a statistically significant relationship was observed between anemia and the absence of severe hematuria in the identification of isolated DN. The ORs were 11.38 (95% CI: 2.51-51.52, $p<0.01$) and 11.66 (1.68-80.96, $p=0.013$), respectively.

Predictive performance of conventional factors with and without anemia for the diagnosis of isolated DN

To assess the significance of anemia in clinically predicting DN, differential diagnostic models were developed using a logistic regression model. Model 1 comprised traditional predictive factors, such as a long duration of DM, DR, and the absence of severe hematuria. Model 2 included anemia and all other factors in model 1. The probability of a diagnosis of isolated DN (p_{DN}) was calculated by two formulas, as follows: 1/[1+EXP(3.03-DM history×0.957-DR×1.291-hematuria×1.623)] in model 1, and 1/[1+EXP(4.239-DM

Table 3. Multivariable Logistic Regression Analyses in the Identification of the Factors Associated with Isolated DN.

	univariable			multivariable		
	OR	95% CI	p value	OR	95% CI	p value
age (per 10-year increase)	0.86	0.62-1.18	0.349	0.62	0.38-1.00	0.052
male	0.63	0.24-1.61	0.331	0.93	0.26-3.39	0.915
long duration of DM (≥10years)	2.43	0.93-6.30	0.069	1.94	0.57-6.60	0.287
diabetic retinopathy	4.39	1.67-11.50	<0.01	2.03	0.62-6.68	0.245
absence of severe hematuria (<30/HPF)	5.30	1.11-25.23	0.036	11.66	1.68-80.96	0.013
nephrotic range proteinuria	2.25	0.88-5.75	0.090	2.92	0.85-10.05	0.087
anemia*	5.71	1.99-16.40	<0.01	11.38	2.51-51.52	<0.01

Multiple regression analysis was performed adjusted for age, sex, long duration of DM, presence of diabetic retinopathy, absence of severe hematuria, nephrotic range proteinuria, and anemia.

*diffinition of anemia: male; Hb<13g/dL, female; Hb<12g/dL.

DN: diabetic nephropathy, DM: diabetes mellitus, HPF: high power field, OR: odds ratio, CI: confidence interval

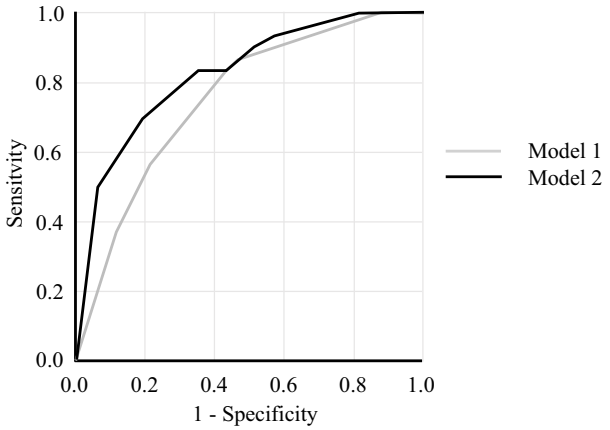

Figure 1. An ROC analysis for the prediction of isolated DN. Model 1 shows the receiver operating characteristics curve obtained from conventional predictive factors consisting of a long duration of diabetes mellitus, diabetes retinopathy (DR), and the absence of severe hematuria. Model 2 shows the factors used in model 1+anemia. p=0.057, log-rank test. ROC: receiver operating characteristic, DN: diabetic nephropathy

history×0.578-DR×1.148-hematuria×2.056-anemia×1.836)] in model 2 [DM history (0<10 years, 1≥10 years); DR (0 no, 1 yes); hematuria (0≥30/HPF, 1<30/HPF); anemia (0 no, 1 yes)]. The ROC curves were illustrated with p_{DN} (Fig. 1).

The AUC of model 1 was 0.755, with 83.3% and 56.9% sensitivity and specificity, respectively, and that of model 2 was 0.826, with 70.0% and 80.4% sensitivity and specificity, respectively. In the analysis of the model predictability, AIC of model 2 was improved to 91.844, relative to 100.152 of model 1, and the NRI was 27.0% (Supplementary material 2), although the difference between their AUCs was not significant in this study. The predictive performance for isolated DN was suggested to have been improved by including anemia.

Pathological characteristics of the study population

The severity of glomerular and tubulointerstitial damage of the kidney biopsy specimens was evaluated, and the results are illustrated in Fig. 2 and Table 4. Glomerular and nodular lesions were more prominent in the isolated DN group than in the NDKD with concurrent DN (p=0.014, multiple comparison corrected using Bonferroni method), whereas the percentage of glomeruli showing global sclerosis did not differ significantly between the groups. In the evaluation of tubulointerstitial lesions, the isolated DN group showed significantly more severe IF/TA than the NDKD group (p<0.05, Fisher's exact probability test). However, interstitial inflammation was more profound in the NDKD group, especially the NDKD alone group, than in other groups. Regarding vascular lesions, arteriolar hyalinosis and arteriosclerosis were widely observed in all groups. In particular, the extent of arteriolar hyalinosis was significant in the isolated DN group. These results suggest that DM-induced chronic renal damage was the most severe lesion in the isolated DN group. Although no glomeruli were obtained in electron microscopy samples from 19 patients, GBM thickening was observed under light microscopy in all patients diagnosed with DN. This was also confirmed in 14 patients in the NDKD alone group, and 9 of these 14 were diagnosed with membranous nephropathy. However, the GBM manifestation may have been early lesions in DN.

Univariable and multivariable logistic regression analyses for the identification of pathological factors associated with anemia

We examined the relationship between pathological findings and anemia using a multivariable logistic regression analysis after adjusting for the age, sex, and each pathological factor (Table 5). This analysis showed that the IF/TA score had a significant association with anemia (OR: 7.31, 95% CI: 2.33-23.00, p<0.01) as well as the glomerular lesion grade (OR: 4.13, 95% CI: 1.46-11.71, p<0.01).

The renal survival and mortality after the kidney biopsy

The Kaplan-Meier curves of the renal survival and mor-

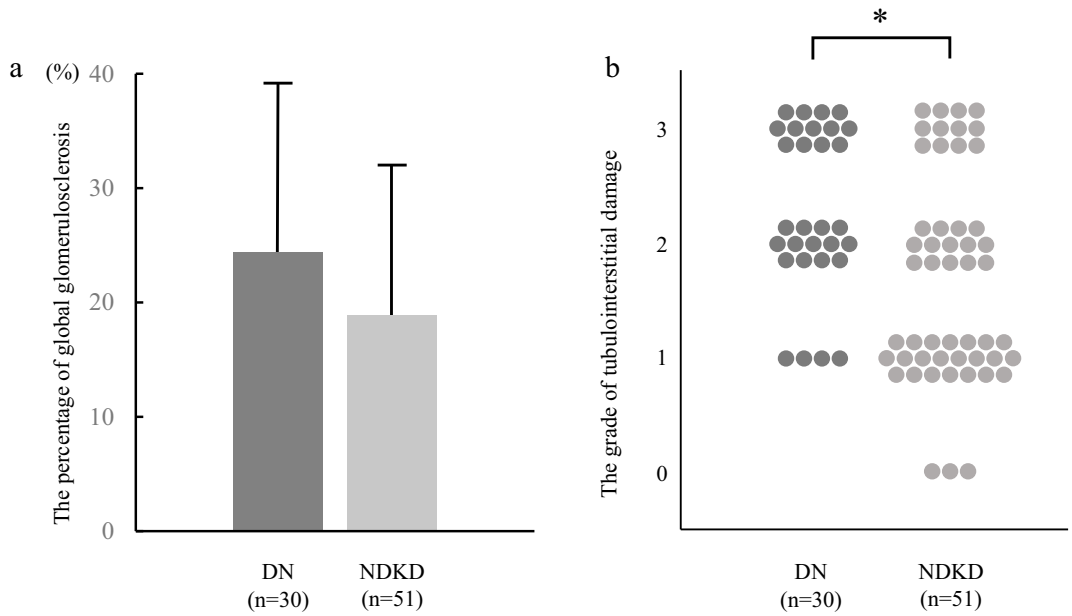

Figure 2. **Glomerular and tubulointerstitial damage of kidney biopsy specimens. a: The percentage of glomeruli that showed global sclerosis in the isolated diabetic nephropathy (DN) group (black line) and the non-diabetic kidney disease (NDKD) group (gray line); the error bar indicates the standard deviation. b: The distribution of the grade of tubulointerstitial damage in the isolated DN group (black dots) and NDKD group (gray dots). *The result of the χ^2 test was statistically significant ($p<0.05$).**

Table 4. **Pathological Characteristics of the Study Population.**

	total (n=81)	NDKD (n=51)		isolated DN (n=30)	p value
		NDKD alone (n=37)	NDKD concurrent DN (n=14)		
nodular lesion [n (%)]	60.4±13.7	2 (3.9)		16 (53.3) [b †]	<0.01
		0 (0) [b]	2 (14.3) [†]		<0.01
glomeruli showed global sclerosis (%)	19.0 (7.4-30.4)	14.3 (6.1-28.6)		21.25 (10.0-35.7)	0.145
		13.2 (5.6-28.6)	19.65 (11.8-29.2)		0.236
IF/TA (score 0/1/2/3)	3/26/27/25	3/22/14/12		0/4/13/13 [b]	<0.01
		3/18/7/9 [b]	0/4/7/3		<0.01
interstitial inflammation (score 0/1/2)	13/57/11	11/30/10		2/27/1 [b]	0.011
		9/20/8 [b]	2/10/2		0.025
arteriolar hyalinosis (score 0/1/2)	11/24/46	10/18/23		1/6/23 [b]	0.013
		10/15/12 [# b]	0/3/11 [#]		<0.01
arteriosclerosis (score 0/1/2)	11/24/46	10/16/25		1/8/21	0.073
		8/11/18	2/5/7		0.190
GBM thickening [n (%)]* male >430 nm, female >395 nm	47 (75.8)	24 (61.5)		23 (100) [b]	<0.01
		14 (48.3) [# b]	10 (100) [#]		<0.01

Renal pathology was evaluated and scored in accordance with the classification of diabetic nephropathy by Renal Pahtology Society.

* n=62 (No glomeruli were obtained in electron microscopy samples from 19 patients).

[#]significant difference between NDKD alone and NDKD concurrent DN ($p<0.017$, Bonferroni method).

[b] significant difference between NDKD alone and isolated DN ($p<0.017$, Bonferroni method).

[†]significant difference between NDKD concurrent DN and isolated DN ($p<0.017$, Bonferroni method).

NDKD: non-diabetic kidney disease, DN: diabetic nephropathy, IF/TA: interstitial fibrosis/tubular atrophy, GBM: glomerular basement membrane

tality are shown in Fig. 3. A total of 62.5% of patients in the isolated DN group developed ESKD, and the proportions of patients who developed ESKD in the NDKD alone and NDKD with concurrent DN groups were 8.8% and 23.5%, respectively (15.5% in NDRD group). Their renal survival rates at 1-, 2-, and 5-years were 85.7%, 78.9%, and 37.5% in the isolated DN group, respectively; 96.8%, 96.2%, and 92.9% in the NDKD alone group, respectively; and 90.0%, 88.9%, and 50.0% in the NDKD with concurrent DN group, respectively (92.6%, 88.9%, and 78.9% in the NDKD

Table 5. Multivariable Logistic Regression Analyses in the Identification of the Pathological Factors Associated with Anemia.

	univariable		maltivariable	
	OR (95% CI)	p value	OR (95% CI)	p value
age (per 10-year increase)	1.38 (0.99-1.90)	0.055	2.13 (1.24-3.68)	<0.01
male	0.30 (0.11-0.83)	0.020	0.56 (0.13-2.32)	0.421
IF/TA score (0/1/2/3)	4.61 (2.99-9.27)	<0.01	7.31 (2.33-23.00)	<0.01
interstitial inflammation score (0/1/2)	3.12 (1.23-7.96)	0.017	0.89 (0.25-3.20)	0.859
arteriolar hyalinosis score (0/1/2)	1.28 (0.70-2.36)	0.428	0.54 (0.19-1.52)	0.242
arteriosclerosis score (0/1/2)	1.56 (0.84-2.89)	0.161	0.38 (0.14-1.05)	0.063
class of glomerular lesion (NDKD or I/II/III/IV)	3.09 (1.65-5.80)	<0.01	4.13 (1.46-11.71)	<0.01

Multiple regression analysis was performed adjusted for age, sex, IF/TA score, interstitial inflammation score, arteriosclerosis score, arteriosclerosis score, and grade of glomerular lesion.

Renal pathology was evaluated and scored in accordance with the classification of diabetic nephropathy by Renal Pahtology Society.

OR: odds ratio, CI: confidence interval, IF/TA: interstitial fibrosis and tubular atrophy, NDKD: non-diabetic kidney disease

group). The renal prognosis was significantly better in the NDKD alone group than in the isolated DN group based on a multiple comparison (p<0.017, Log-rank test after Bonferroni correction), whereas the mortality showed no significant difference.

Discussion

In this study, diabetic patients who underwent a kidney biopsy in our institute were reviewed, and biopsy-proven DN cases were found to have a higher prevalence of anemia than the NDKD group. The AIC and NRI analyses suggested the existence of improvement in the predictive performance following the addition of anemia to the previously demonstrated factors. A further histopathological analysis revealed that the tissue of patients in the DN group showed more severe IF/TA and arteriolar hyalinosis, regardless of the class of glomerular lesion, than NDKD alone group, whereas no significant difference was observed in the percentages of global glomerulosclerosis. Furthermore, IF/TA had the strongest association with anemia according to a multiple regression analysis.

The major morphological features of DN resulted from alternations in the extracellular matrix, glomerular basement membrane thickening, increases in the glomerular mesangial matrix, and tubulointerstitial injury. These findings showed that tubulointerstitial injuries could develop in the early stage of DN. Fioretto et al. (14) investigated 34 consecutive renal biopsies in microalbuminuric non-insulin-dependent diabetic patients, and observed that 41.2% of biopsies showed atypical patterns of injury, with no or only mild diabetic glomerular changes associated with severe tubulointerstitial injuries. There are multiple pathways associated with glomerular and tubulointerstitial injuries, such as hemodynamic alternations caused by arteriolar resistance, oxidative stress, cell signaling and transcription factors, and pro-inflammatory molecules (15). Proximal tubular epithelial cells are an important target of the aforementioned abnormalities, and cell cycle arrest, cellular hypertrophy, senescence, and inflammatory and profibrotic cytokine secretions are suggested as down-stream effects (16) that can result in the development of IF/TA independent of global glomerulosclerosis.

Renal tubulointerstitial injuries are reportedly associated with anemia. The mechanism has been shown to involve erythropoietin-producing fibroblasts undergoing transformation into myofibroblasts in fibrotic kidneys *in vivo* (17). Furthermore, some human studies have shown that interstitial damage and relative erythropoietin deficiency leading to anemia occur in the early stage of DN (18, 19). These results suggest that DM-associated severe IF/TA (compared with NDKD) impaired erythropoietin production, resulting in earlier anemia, independent of the glomerular injuries and renal function.

In our study, prognosis after the biopsy was evaluated. The isolated DN group showed a significantly poorer renal survival rate than the NDKD group. The existence of established disease-specific treatments for each disease may have improved the prognosis in the NDKD group. However, patients with NDKD with concurrent DN tended to have a poor renal prognosis, even in the NDKD group. This may suggest that renal damage is strongly affected by histological changes in DN. Okada et al. (20) reported that interstitial lesions but not glomerular lesions were a significant predictor of the renal prognosis in diabetic nephropathy patients with type 2 diabetes and overt proteinuria. Furthermore, anemia was found to be associated with poor renal outcomes in CKD (21, 22). Generally, there appears to be a strong correlation among the renal prognosis, anemia, and interstitial injury.

In addition, anemia can aggravate renal fibrosis by causing renal tissue hypoxia via the stimulation of cytokine production, including hypoxia-inducing factor (HIF)-1. Recently, a novel oral inhibitor of HIF-prolyl hydroxylase (HIF-PH) became clinically available, alongside conventional therapy with ESAs. The renoprotective effect of the

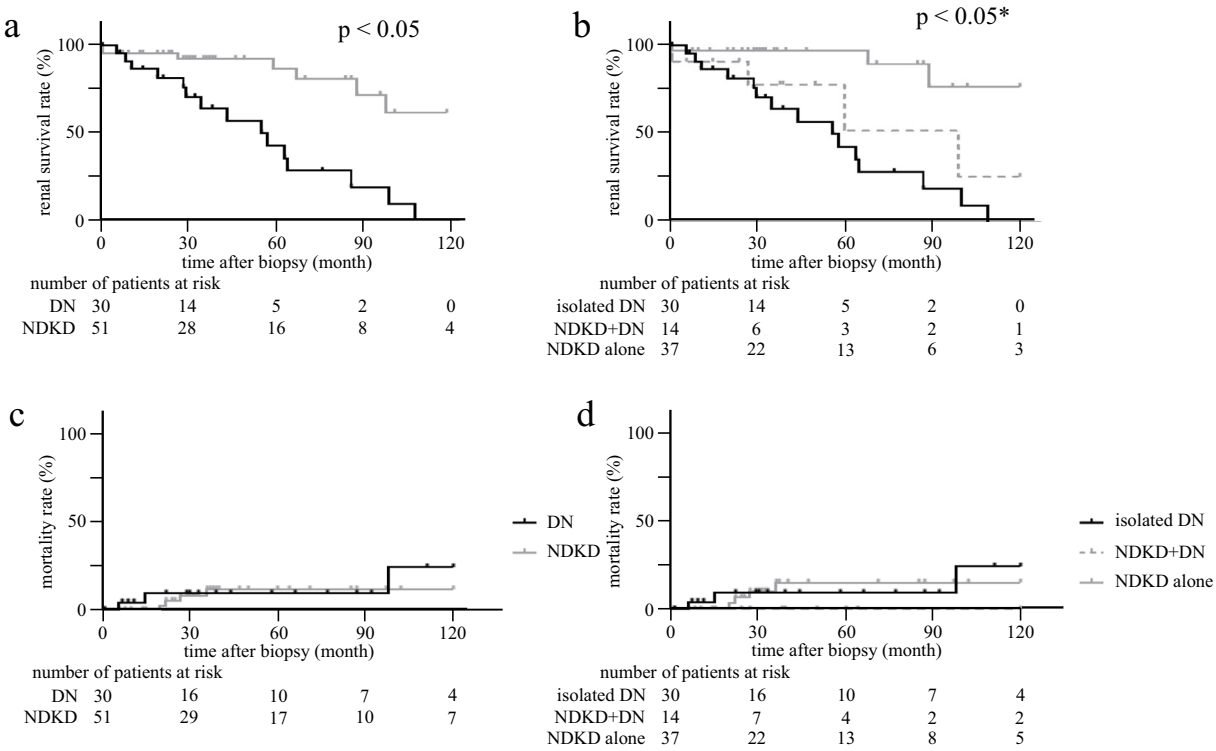

Figure 3. Relationship between the renal pathology and the renal survival (a, b), and mortality (c, d). a, c: Kaplan-Meier curve according to the isolated DN group (black line) and NDKD group (gray line). b, d: Kaplan-Meier curve according to the isolated DN (black solid line), NDKD with concurrent DN (NDKD+DN, gray dashed line), and NDKD alone (gray solid line) groups. *There was a significant difference in the comparison between the NDKD alone and isolated DN groups (p<0.017, Log rank test after Bonferroni correction). DN: diabetic nephropathy, NDKD: non-diabetic kidney disease

HIF-PH inhibitor remains unknown; however, Wakashima et al. (23) conducted an *in vitro* study and showed that HIF-PH inhibitor suppressed the expression of fibroblast growth factors 2, 7, and 18, which are upregulated during the transformation of renal interstitial fibroblasts. Thus, HIF-PH inhibitors might have therapeutic potential in the management of tubular interstitial fibrosis alongside their stimulatory effect of erythropoietin production.

The strength of this study was that we focused on anemia as a predictive factor for DN and compared its association with the kidney function and IF/TA. The measurement of hemoglobin is a clinically available and cost-effective marker, and anemia is an important therapeutic target in CKD, as described in the clinical practice guideline in Japan (24). Novel therapy using an HIF-PH inhibitor can be applied along with conventional therapy of ESAs and iron supplements.

However, several limitations associated with the present study also warrant mention, such as the retrospective nature, small sample size compared to many other studies, and potential selection bias, including the small number of isolated DN cases. Furthermore, not all reported parameters could be analyzed because of some missing data. In particular, data on pro-inflammatory cytokines and anemia-related factors, such as high-sensitivity C-reactive protein, erythropoietin, and others, may help elucidate the mechanisms underlying anemia in patients with DN. Thus, this study lacks the appropriate statistical power, and the results should be confirmed in a larger study population that includes patients of various ethnic backgrounds.

In summary, DN is associated with severe anemia and IF/TA, and anemia helps clinicians discriminate clinically between isolated DN and NDKD. At present, a kidney biopsy is considered in diabetic patients who are likely to develop concurrent NDKD. However, novel pharmacotherapies may cause a significant delay in the progression of DN, and anemia is an important therapeutic target. To confirm the potential utility of anemia as a predictor and therapeutic target, a large-scale clinical study with biopsy-proven DN cases is necessary. In this regard, a kidney biopsy for diabetic patients should be considered more aggressively. The results will be useful for the intensive management of diabetic patients suffering from kidney disease and for improving their clinical outcomes.

Written informed consent was obtained from all individual participants included in the study.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

We would like to thank Satoshi Hisano (deceased) for his pathological evaluation of the biopsy specimens.

Kenji Ito and Soichiro Yokota contributed equally to this work.

References

1. Nitta K, Masakane I, Hanafusa N, et al. 2018 Annual Dialysis Data Report, JSDT Renal Data Registry. Nihon Toseki Igakkai Zasshi (Jpn J Dial Ther) **52**: 679-754, 2019 (in Japanese, Abstract in English).
2. Kritmetapak K, Anutrakulchai S, Pongchaiyakul C, Puapairoj A. Clinical and pathological characteristics of non-diabetic renal disease in type 2 diabetes patients. Clin Kidney J **11**: 342-347, 2018.
3. Liang S, Zhang XG, Cai GY, et al. Identifying parameters to distinguish non-diabetic renal diseases from diabetic nephropathy in patients with type 2 diabetes mellitus: a meta-analysis. PLoS One **8**: e64184, 2013.
4. Liu D, Huang T, Chen N, et al. The modern spectrum of biopsy-proven renal disease in Chinese diabetic patients-a retrospective descriptive study. PeerJ **6**: e4522, 2018.
5. Soni SS, Gowrishankar S, Kishan AG, Raman A. Non diabetic renal disease in type 2 diabetes mellitus. Nephrology (Carlton) **11**: 533-537, 2006.
6. Wang J, Han Q, Zhao L, et al. Identification of clinical predictors of diabetic nephropathy and non-diabetic renal disease in Chinese patients with type 2 diabetes, with reference to disease course and outcome. Acta Diabetol **56**: 939-946, 2019.
7. Jiang S, Fang J, Yu T, et al. Novel model predicts diabetic nephropathy in type 2 diabetes. Am J Nephrol **51**: 130-138, 2020.
8. Yang Z, Feng L, Huang Y, Xia N. A differential diagnosis model for diabetic nephropathy and non-diabetic renal disease in patients with type 2 diabetes complicated with chronic kidney disease. Diabetes Metab Syndr Obes **12**: 1963-1972, 2019.
9. Astor BC, Muntner P, Levin A, Eustace JA, Coresh J. Association of kidney function with anemia: the Third National Health and Nutrition Examination Survey (1988-1994). Arch Intern Med **162**: 1401-1408, 2002.
10. El-Achkar TM, Ohmit SE, McCullough PA, et al. Higher prevalence of anemia with diabetes mellitus in moderate kidney insufficiency: The Kidney Early Evaluation Program. Kidney Int **67**: 1483-1488, 2005.
11. Matsuo S, Imai E, Horio M, et al. Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis **53**: 982-992, 2009.
12. Shimamoto K, Ando K, Fujita T, et al. The Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2014). Hypertens Res **37**: 253-390, 2014.
13. Tervaert TW, Mooyaart AL, Amann K, et al. Pathologic classification of diabetic nephropathy. J Am Soc Nephrol **21**: 556-563, 2010.
14. Fioretto P, Mauer M, Brocco E, et al. Patterns of renal injury in NIDDM patients with microalbuminuria. Diabetologia **39**: 1569-1576, 1996.
15. Lim A. Diabetic nephropathy - complications and treatment. Int J Nephrol Renovasc Dis **7**: 361-381, 2014.
16. Tang SC, Lai KN. The pathogenic role of the renal proximal tubular cell in diabetic nephropathy. Nephrol Dial Transplant **27**: 3049-3056, 2012.
17. Asada N, Takase M, Nakamura J, et al. Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosis and renal anemia in mice. J Clin Invest **121**: 3981-3990, 2011.
18. Bosman DR, Winkler AS, Marsden JT, Macdougall IC, Watkins PJ. Anemia with erythropoietin deficiency occurs early in diabetic nephropathy. Diabetes Care **24**: 495-499, 2001.
19. Winkler AS, Marsden J, Chaudhuri KR, Hambley H, Watkins PJ. Erythropoietin depletion and anaemia in diabetes mellitus. Diabet Med **16**: 813-819, 1999.
20. Okada T, Nagao T, Matsumoto H, Nagaoka Y, Wada T, Nakao T. Histological predictors for renal prognosis in diabetic nephropathy in diabetes mellitus type 2 patients with overt proteinuria. Nephrology (Carlton) **17**: 68-75, 2012.
21. Hayashi T, Uemura Y, Kumagai M, Kimpara M, Kanno H, Ohashi Y. Effect of achieved hemoglobin level on renal outcome in non-dialysis chronic kidney disease (CKD) patients receiving epoetin beta pegol: MIRcerA CLinical Evidence on Renal Survival in CKD patients with renal anemia (MIRACLE-CKD Study). Clin Exp Nephrol **23**: 349-361, 2019.
22. Ushio Y, Kataoka H, Sato M, et al. Association between anemia and renal prognosis in autosomal dominant polycystic kidney disease: a retrospective study. Clin Exp Nephrol 2020.
23. Wakashima T, Tanaka T, Fukui K, et al. JTZ-951, an HIF prolyl hydroxylase inhibitor, suppresses renal interstitial fibroblast transformation and expression of fibrosis-related factors. Am J Physiol Renal Physiol **318**: F14-F24, 2020.
24. Japanese Society of Nephrology. Essential points from Evidence-based Clinical Practice Guidelines for Chronic Kidney Disease 2018. Clin Exp Nephrol **23**: 1-15, 2020.