Clinical and Pathological Significance of Autoantibodies to Erythropoietin Receptor in Type 2 Diabetic Patients With CKD

Clinical and pathological significance of autoantibodies to erythropoietin receptor in type 2 diabetic patients with chronic kidney disease

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A running headline: Anti-EPOR antibodies in diabetic nephropathy

Word Counts:

Abstract word count: 218 words

Text word count: 3752 words (excluding references, tables, and figures)

Abstract

Introduction: We examined the impact of autoantibodies to the erythropoietin receptor (EPOR) in type 2 diabetic patients with chronic kidney disease (CKD).

Methods: One hundred and twelve Japanese patients with type 2 diabetes who had CKD were enrolled in this study and followed for a mean of 45 months. Sera from those patients were screened for anti-EPOR antibodies using enzyme-linked immunosorbent assays. Results: Anti-EPOR antibodies were detected in 26 patients (23%). Anti-EPOR antibodies were associated with low hemoglobin concentrations and decreased renal function. In patients with biopsy-proven diabetic nephropathy, anti-EPOR antibodies were associated with increased levels of interstitial inflammation. A decrease in renal function was observed more frequently in patients with antibodies than in patients without antibodies, and the presence of the antibodies as well as well-known clinical parameters, including proteinuria and low glomerular filtration rate, was a significant risk factor for end-stage renal disease (ESRD). In human tubular epithelial cells, HK-2, immunoglobulin G fractions with anti-EPOR antibodies upregulated the expression of monocyte chemoattractant protein-1 mRNA under high glucose concentration.

Conclusion: The present study suggests that anti-EPOR antibodies might be involved in the progression of renal lesions, as well as in the impaired erythropoiesis in type 2

diabetic patients with CKD. Further, the presence of anti-EPOR antibodies may be an additional predictor for ESRD in type 2 diabetes.

Keywords: diabetic nephropathy, erythropoietin receptor, autoantibodies, prognosis, risk factor

Introduction

Diabetes and its complications are major causes of morbidity and mortality in most countries.[1] Among diabetic complications, nephropathy occurs in 20–40% of patients during the course of their disease.[2] Although kidney disease attributable to diabetes is referred to as diabetic nephropathy or diabetic kidney disease, diabetes and various kidney diseases have become common chronic conditions.[3] Thus, the prevalence of chronic kidney disease (CKD) is increasing in proportion to the increase in the prevalence of diabetes, and it has been predicted to continue to increase in the future.[4] Of note, diabetes is a risk factor of cardiovascular disease and death, and CKD further increases these risks.[5-8] Anemia is observed commonly in diabetic patients with CKD and is involved in the increased risk for the progression of kidney disease and cardiovascular mortality and morbidity.[7, 9-11]

Anemia in diabetic patients with CKD may result from one or more mechanisms. Among these, the major causes are iron and erythropoietin (EPO) deficiencies and hyporesponsiveness to the action of EPO.[11] In view of EPO hyporesponsive anemia, we previously detected and reported autoantibodies to the EPO receptor (EPOR) as a possible cause of anemia with erythroid hypoplasia.[12] In this study, unexpectedly, these antibodies were detected even in diabetic patients with CKD,

although their clinical significance remains to be investigated.[12] In addition, a recent study revealed that anti-EPOR antibodies were associated with overall disease activity and decline of renal function in patients with systemic lupus erythematosus.[13]

These results prompted us to examine the clinical and pathological impact of anti-EPOR antibodies in type 2 diabetic patients with CKD. We found that anti-EPOR antibodies are detected in a subset of patients, are associated with renal lesions, and that they are inversely related to the preservation of renal function.

Methods

Patients

One hundred and twelve type 2 diabetic patients who had been diagnosed as having CKD and followed at Kanazawa University Hospital between 1989 and 2014 were included in this study. The mean follow-up period was 45.3 ± 47.4 months. Patients with secondary diabetes, renal transplantation, or dialysis were excluded. Among the enrolled patients, 51 underwent a renal biopsy. A diagnosis of diabetic nephropathy was confirmed by histological characteristics using renal biopsy specimens including light microscopy, electron microscopy, and immunofluorescence examination. Renal biopsy was performed for the precise diagnosis of renal lesions with the consent of each patient. All blood samples were obtained after the patients had given their written informed consent at admission for the renal biopsy or for work up and treatment of the disease. The study protocol adhering to the Declaration of Helsinki was approved by the medical ethics committee of Kanazawa University. Of note, 8 healthy individuals in addition to 40 ones were included as healthy controls, and as a result, anti-EPOR antibodies were not detected in serum samples from these subjects.[12]

Clinical features and routine laboratory tests

Demographic and clinical features were evaluated for each enrolled patient. Baseline clinical and laboratory findings including the use of angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs) and erythropoiesis stimulating agents (ESAs) were extracted from medical records. Twenty-four hour urinary protein excretion, serum creatinine, estimated glomerular filtration rate (eGFR), HbA1c, body mass index (BMI), systolic and diastolic blood pressure, total cholesterol, hemoglobin (Hb), iron, total iron binding capacity (TIBC), ferritin, and C-reactive protein (CRP) were used as baseline clinical parameters at admission. eGFR for Japanese patients was calculated using the following equation: eGFR (mL/min/1.73 m^2) = 194 × serum creatinine$^{-1.094}$ × age$^{-0.287}$ (if female, × 0.739).[14] HbA1c levels were presented as National Glycohemoglobin Standardization Program values according to the recommendations of the Japan Diabetic Society[15] and International Federation of Clinical Chemistry values. Autoantibodies against glutamic acid decarboxylase (GAD) were measured by a commercial quantitative enzyme-linked immunosorbent assay (ELISA; SRL, Inc., Tokyo, Japan).

Detection of autoantibodies to EPOR

Anti-EPOR antibodies were detected by ELISA as described previously.[12] Briefly, polyvinyl 96-well microtitration plates (Nunc International, Tokyo, Japan) were coated with recombinant human EPOR (R & D Systems, Minneapolis, MN, USA) at 5 μg/mL diluted in 0.2 M $NaHCO_3$ buffer at 4 °C for 24 h. The remaining free-binding sites were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 4 °C. After the plates were washed with Tween 20-Tris-buffered saline, the samples were added in duplicate at 1:1,000 dilution to 1% BSA in PBS for 20 h at 4 °C. The plates were washed 4 times with the same buffer and incubated with goat anti-human Ig-conjugated with horseradish peroxidase (Millipore, Temecula, CA, USA) at 1:5,000 dilution for 1.5 h at room temperature. The substrate tetramethylbenzidine (KPL, Gaithersburg, MD, USA) was added and the reaction was stopped by the addition of 2 N sulfuric acid. The optical density at 450 nm (OD_{450}) was determined by an automatic plate reader and the sample was considered to be antibody-positive when the ratio of patient serum OD_{450} to that of normal control sera was ≥2.4. This cutoff OD value was determined by a preliminary analysis using a receiver operating characteristic curve to predict, sensitively and specifically, renal outcome among the enrolled type 2 diabetic patients with a positive OD value ≥ 1.5[12] (data not shown).

Purification of immunoglobulin G fractions

Sera from patients and control subjects were clarified by centrifugation at 1,500 × *g* for 20 min (500 µL) and filtration through 0.45 µm filters (Millipore, County Cork, Ireland). Immunoglobulin (Ig) G fractions were prepared using a MAb Trap Kit (GE Healthcare, Tokyo, Japan) according to the manufacturer's instructions. Purified IgG fractions were concentrated using Centriprep centrifugal filters (Millipore) and stored at 4°C until required.

Histopathologic studies

Renal biopsy specimens were prepared for light microscopic examination as described previously.[7] In brief, patients' samples were fixed in 10% phosphate-buffered formalin (pH 7.2), embedded in paraffin, and sliced into 4-µm sections. These specimens were stained with hematoxylin and eosin, periodic acid Schiff (PAS) reagent, Mallory-Azan, and periodic acid silver methenamine, and were examined under a light microscope. The severity of diffuse lesions of glomeruli was graded on a scale of 0 to 4 as described previously: grade 0, all glomeruli appear normal; grade 1, local lesions present within each glomerulus and focal lesions present within the kidney; grade 2, mesangial thickening is diffuse within the glomerulus and generalized throughout the kidney;

grade 3, capillary lumina are narrowed and obliterated only locally; and grade 4, the lumen is generally narrowed and the entire glomerulus is ischemic and appears to be hyalinized.[7] Nodular lesions, exudative lesions, and mesangiolysis were simply shown as their presence or absence in each specimen.[7] The severity of interstitial fibrosis and tubular atrophy (IFTA) and interstitial inflammation was scored according to the criteria of the Renal Pathology Society (RPS).[16] The severity of IFTA was evaluated and graded on a scale from 0 to 3: grade 0, no IFTA; grade 1, <25%; grade 2, 25–50%; and grade 3, >50%.[16] The severity of interstitial inflammation was evaluated and graded on a scale from 0 to 2: grade 0, absent; grade 1, infiltration only in relation to IFTA; and grade 2, infiltration in areas without IFTA.[16] The severity of arteriolar hyalinosis was evaluated and graded on a scale from 0 to 3 as described previously: grade 0, normal appearance without PAS-positive deposits; grade 1, a light PAS-positive thickening is observed but at less than half the circumference of the arteriole in many arterioles; grade 2, most vessel walls are moderately thickened with PAS-positive deposition without apparent luminal narrowing; and grade 3, a heavy thickening of the majority of the vessel walls is seen with luminal narrowing or obliteration.[7] The severity of arteriosclerosis was evaluated and graded on a scale from 0 to 2 according to the RPS criteria: grade 0, no intimal thickening; grade 1, intimal thickening less than the thickness of the media; and

grade 2, intimal thickening greater than the thickness of the media.[16] Renal tissue specimens were examined by four nephrologists.

Renal outcome

The outcome for this study was end-stage renal disease (ESRD), which was defined as the need for dialysis or renal transplantation, as described elsewhere.

Cells and cell culture

The human renal tubular epithelial cell HK-2 was grown according to the manufacturer's instructions (ATCC, CRL-2190, Manassas, VA). To examine the effect of purified IgG fractions with anti-EPOR antibodies on function of HK-2 cells under diabetic conditions, HK-2 cells were incubated with regulated concentrations of D-glucose (Wako chemicals Inc. Tokyo, Japan) and/or EPO. Briefly, HK-2 cells (3×10^5/ml) were cultured in DMEM supplemented with 0.5% heat-inactivated FBS (Gibco BRL) at 37℃ in a humidified atmosphere with 5% CO_2 for 24 h after the isolation in the presence of 5 or 20 mmol/l of D-glucose. Further, IgG fractions with anti-EPOR antibodies from patients were applied. HK-2 cells (1.5×10^5/ml) were preincubated with IgG (10 μg) in each culture medium for 60 min. Subsequently,

HK-2 cells were incubated in the presence of recombinant human EPO (10 ng/ml) (Thermo Fisher Scientific Inc., Yokohama, Kanagawa, Japan) for 48 h.

RT-PCR

The transcript of human EPOR was detected using reverse transcription-polymerase chain reaction (RT-PCR). In brief, total RNA was extracted from cultured HK-2 cells under normal or high concentration of glucose, and the complementary DNA (cDNA) was reverse-transcribed from 1μg total RNA using a RT-PCR kit (Takara Shuzo, Tokyo, Japan). The cDNA product was amplified by PCR as follows; incubation for three minutes at 94℃, followed by 35 cycles of one minute at 94℃, two minutes at 55℃, and three minutes at 72℃and a final extension for seven minutes at 72℃[17]. Primers for human EPOR (sense, 5'-GCA-CCG-AGT-GTG-TGC-TGA-CGA-A-3', antisense, 5'-GGT-CAG-CAG-CAC-CAG-CAT-GAC-3') were used to examine expression of human EPOR[31]. The human EPO-dependent cell line AS-E2[12, 18] was used as a positive control. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for PCR controls[19].

Real-time quantitative PCR

To determine transcripts of MCP-1, total RNA was extracted from cultured HK-2 cells as described above. Quantitative real-time RT-PCR was performed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Assay IDs of TaqMan gene expression assays were Hs00234140 for MCP-1 and Hs99999905 for GAPDH.

Statistical analysis

All comparisons between the two patient groups were performed using the chi-square test and Mann-Whitney's U test. A renal outcome curve was obtained using the Kaplan-Meier method and compared by using the log-rank test. A multivariate Cox proportional hazards regression model was used to select factors that significantly affected the incidence of renal outcome and to estimate risk. The following variables were incorporated as covariates: age, sex, BMI, systolic blood pressure, proteinuria, eGFR, HbA1c, Hb, and the presence of anti-EPOR antibodies. A multiple comparison among three groups in *in vitro* experiments was performed using the Tukey's test. All analyses were carried out using the statistical package SPSS, version 22 (SPSS, Tokyo, Japan) and the Stata 14.2 software (Stata Corporation, College Station, Tex., USA). *P*-values less than 0.05 were considered statistically significant.

Results

Characteristics of the enrolled patients

None of 112 patients enrolled had anti-GAD antibodies. The baseline characteristics of the 112 patients are shown in Table 1. Patients' mean age was 62.9 years, and 76 (67.9%) were male. The mean amount of urinary protein excretion was 2.7 g/day. The mean serum creatinine concentration was 2.4 mg/dL, and the mean eGFR was 42.0 mL/min/1.73 m^2. The median Hb concentration and reticulocyte count were 11.2 g/dL and 4.4×10^4/mL, respectively. Diabetes-related parameters included a mean duration of 13.6 years at the time of work up on admission and a mean HbA1c of 6.7%. The mean levels of serum iron, TIBC, and ferritin were 71 mg/dL, 244 mg/dL, and 233 ng/mL, respectively. The mean CRP level was 0.6 mg/dL.

Clinical characteristics of type 2 diabetic patients with and without anti-EPOR antibodies

Patients' serum samples were analyzed by ELISA. Anti-EPOR antibodies were detected in 26 of the 112 patients. Demographic and clinical findings were compared between the patients with and without anti-EPOR antibodies; selected

findings are shown in Table 1. Patients with the antibodies were older than those without the antibodies (67.5 vs. 61.5 years, respectively). While the extent of proteinuria did not differ between both groups, renal function was significantly lower in the patients with the antibodies than in those without the antibodies, as indicated by the mean serum creatinine concentration and eGFR. In addition, the levels of total cholesterol and triglyceride were significantly decreased in the patients with anti-EPOR antibodies compared to those without the antibodies. The Hb level was significantly lower in the patients with anti-EPOR antibodies than in those without the antibodies, though the number of reticulocytes was not significantly different. ESAs were used more frequently in patients with the antibodies than those without (64 % vs. 19 %, $p < 0.01$). No significant difference was found between the two groups for the other parameters examined, including duration of diabetes, HbA1c, systolic and diastolic blood pressure, HDL-cholesterol, iron, TIBC, ferritin, and CRP at the time of work up on admission and the use of ACE inhibitors or ARBs.

Stratified analysis of enrolled patients into 2 groups according to the baseline eGFR levels revealed that while mean baseline age as well as eGFR was similar in both groups, difference in BMI and Hb levels between patients with and without the antibodies was still observed in those with eGFR below 30 mL/min/1.73m^2 (Supplementary Table 1).

In addition, ESA was more frequently used in patients with the antibodies than those without in this subpopulation (94% vs. 45%).

Renal pathological characteristics of type 2 diabetic patients with anti-EPOR antibodies

Renal biopsy findings were evaluated among the enrolled patients. Among the 51 patients who had been diagnosed histologically with diabetic nephropathy, an interstitial inflammation score of 2 was observed more frequently in the patients with anti-EPOR antibodies than in those without the antibodies, while glomerular and vascular lesions were not significantly different between both groups (Table 2).

Outcome of renal function

Follow-up data were available for renal events in the enrolled 112 patients. The mean follow-up period was 45.3 ± 47.4 months during the study period. The event-free rate of renal events in the patients with anti-EPOR antibodies was significantly lower than in those without the antibodies (Figure 1). When stratified into 2 groups according to the baseline eGFR, the difference was observed in patients with baseline eGFR below 30 mL/min/1.73m^2, while it was not in those above 30

mL/min/1.73m^2 (Supplementary Figure 1A and B). The difference in renal survival was also observed in patients with baseline eGFR below 60 mL/min/1.73m^2, while there was a statistical difference in baseline eGFR (Supplementary Figure 2A and B, Supplementary Table 2).

Clinical parameters associated with renal events

The results of multivariate Cox proportional hazards regression analysis are shown in Table 3. The presence of anti-EPOR antibodies as well as high systolic blood pressure, proteinuria and eGFR were independent risk factors for ESRD.

Effects of IgG fractions with anti-EPOR antibodies on the expression of monocyte chemoattractant protein (MCP)-1 mRNA in HK-2 cells under diabetic conditions

The effect of purified IgG fractions with anti-EPOR antibodies on the expression of MCP-1 mRNA in HK-2 cells under a high glucose condition was examined *in vitro*. The expression of EPOR was confirmed by RT-PCR (Figure 2A). Administration of 10 ng/mL EPO suppressed the high glucose-induced upregulation of MCP-1 mRNA (Figure 2B). Further, when compared with IgG purified from a healthy control, IgG from sera of diabetic patients positive for anti-EPOR antibodies in the presence of EPO

upregulated MCP-1 mRNA expression under the high glucose concentration (Figure 2C).

Discussion

The present retrospective study demonstrated that serum anti-EPOR antibodies were detected in a subset of type 2 diabetic patients with CKD. Pathologically, the presence of antibodies was associated with increased interstitial inflammation. In addition, renal function declined more rapidly in patients with the antibodies than in those without the antibodies, and the presence of anti-EPOR antibodies was an additional risk factor for the progression of renal dysfunction.

The present study revealed that anti-EPOR antibodies were present in 23% of type 2 diabetic patients with CKD who did not have anti-GAD antibodies. The presence of autoantibodies to self-proteins in type 2 diabetes has been suggested and reported previously. For example, 11 autoantibodies have been identified by the use of a high-density protein microarray among type 2 diabetic subjects in which the estimated prevalence was highest for autoantibodies to transaldolase 1 and mitochondrial ribosomal protein S7 (79% and 40%, respectively).[20] In addition, autoantibodies to DNA-AGE[21] and apolipoprotein A-1[22] and B-100[23] have also been detected. Among these, anti-apolipoprotein A-1 autoantibodies were present in up to 36% of patients with type 2 diabetes and cardiovascular disease.[22] Although the detailed pathogenic mechanisms underlying the development of autoantibodies remain to be investigated,

anti-EPOR antibodies may be additional autoantibodies that could be present in patients with type 2 diabetes and CKD.

Anti-EPOR antibodies were pathologically associated with renal interstitial inflammation in type 2 diabetic patients with CKD. The interstitial cell infiltration observed in diabetic kidneys is an important pathological finding because of its impact on renal prognosis,[7, 24] as is also found in other etiologies of CKD.[25] Inflammatory interstitial infiltrates include T lymphocytes and macrophages.[24, 25] These inflammatory cells, tubular epithelial cells, and vascular endothelial cells reportedly express EPOR, and the proliferation and activation of T lymphocytes were modulated by EPO administration in previous studies.[26-29] In the present study, EPOR mRNA was detected in human proximal tubular cells, and EPO attenuated the expression of MCP-1 mRNA under high glucose conditions *in vitro*. In addition, IgG fraction with anti-EPOR antibodies in the presence of EPO upregulated the expression of MCP-1 mRNA. Whether binding of anti-EPOR antibodies to EPOR between these cells occurs in the kidney and whether the consequent nephrotoxicities are involved in the progression of renal damage via increased interstitial inflammation, even at areas without IFTA, remain to be investigated. However, anti-EPOR antibodies could be a useful biomarker for interstitial inflammation, which is associated with renal prognosis, in patients with

type 2 diabetes.

The present study revealed that renal function in patients with anti-EPOR antibodies declined earlier than that in patients without the antibodies. Further, in multivariate analysis, the presence of anti-EPOR antibodies was selected as a significant risk factor for the decline of renal function. In this study, clinical parameters including higher proteinuria, and systolic blood pressure, together with lower renal function were confirmed as independent risk factors for ESRD in type 2 diabetic patients.[7, 9, 10] In addition to the effect of anemia on the kidney, the present study and previous basic studies have demonstrated that EPOR is expressed by resident renal cells, including endothelial cells and tubular epithelial cells.[28, 29] Previous studies demonstrated that EPO has renoprotective effects through suppression of inflammation, oxidative stress, and apoptosis of involved cells.[30] On the basis of these non-erythropoietic activities of EPO, the presence of antibodies to EPOR was selected and considered as an independent explanatory factor from the levels of Hb in the present analysis. In the clinical setting, although clinical efficacy has yet to be validated, the administration of ESA to patients with CKD has the potential to preserve renal function, as suggested by previous clinical and epidemiological studies.[31-33] The findings of these studies suggest that renal function may be exacerbated by the blockade of the EPO–EPOR interaction

via antibodies. Taken together, while the effect is relatively limited, anti-EPOR antibodies can serve as a serologic marker for the progression of renal injury in type 2 diabetic patients with CKD in combination with other known parameters including albuminuria and proteinuria.

We noted several limitations in the present study. First, this study was dependent on collectable medical records because of its retrospective design. Second, limiting study enrollment to only patients that were admitted to our tertiary medical care institution for work-up and treatment of an increased amount of proteinuria and/or kidney dysfunction during the course of diabetes, likely created a bias. Third, treatment contents were not evaluated fully. These limitations may have placed significant constraints on the interpretation of the results, particularly those related to differences in renal outcome. However, clinical examinations concomitant with renal biopsy samples in these patients are important for understanding the pathophysiology of type 2 diabetes and its clinical outcome.

In conclusion, autoantibodies to EPOR may be involved in disease progression and may be a useful serologic marker for renal prognosis in type 2 diabetic patients with CKD. These potential biomarker could help physicians diagnose high-risk patients and care them intensively to prevent ESRD.

Disclosure

None.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Number JP16K09608 and a Grant-in-Aid for Practical Research Project for Renal Diseases, from the Japan Agency for Medical Research and Development (Grant Number:15ek0310003h0001).

Contributions of the authors are as follows; A.H. performed the experiments and was involved in the interpretation of the results and preparation of the manuscript. K.F. assisted in the analysis. A.K. contributed to collecting clinical data. H.Y. and T.T. performed and assisted measurement of the antibodies by ELISA. T.T. also performed PCR. Y.I., N.S., S.K. and H.N. assisted in the analysis and were involved in the interpretation of results. M.S. contributed to collection of pathological data and to interpretation of results. T.W. initiated, organized and designed the study, contributed to analysis and interpretation of the data and wrote the manuscript.

References

1. International Diabetes Federation. IDF Diabetes around the world. *DIABETES ATLAS Seventh Edition*; 2015:14-16.
2. American Diabetes Association. Standards of medical care in diabetes-2015. *Diabetes Care* 2015;38 (Suppl. 1):S58-S60.
3. Tuttle KR, Bakris GL, Bilous RW, et al. Diabetic kidney disease: a report from an ADA Consensus Conference. *Am J Kidney Dis* 2014;64:510-533.
4. De Boer IH, Rue TC, Hall YN, et al. Temporal trends in the prevalence of diabetic kidney disease in the United States. *JAMA* 2011;305:2532-2539.
5. Adler AI, Stevens RJ, Manley SE, et al.; UKPDS GROUP. Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64). *Kidney Int* 2003;63:225-232.
6. Wada T, Haneda M, Furuichi K, et al.; Research Group of Diabetic Nephropathy, Ministry of Health, Labour, and Welfare of Japan. Clinical impact of albuminuria and glomerular filtration rate on renal and cardiovascular events, and all-cause mortality in Japanese patients with type 2 diabetes. *Clin Exp Nephrol* 2014;18:613-620.
7. Shimizu M, Furuichi K, Toyama T, et al.; Kanazawa Study Group for Renal

Diseases and Hypertension: Long-term outcomes of Japanese type 2 diabetic patients with biopsy-proven diabetic nephropathy. *Diabetes Care* 2013;36:3655-3662.

8. Toyama T, Furuichi K, Ninomiya T, et al. The impacts of albuminuria and low eGFR on the risk of cardiovascular death, all-cause mortality, and renal events in diabetic patients: meta-analysis. *PLoS One* 2013;8:e71810.
9. Keane WF, Brenner BM, de Zeeuw D, et al.; RENAAL Study Investigators. The risk of developing end-stage renal disease in patients with type 2 diabetes and nephropathy: the RENAAL study. *Kidney Int* 2003;63:1499-1507.
10. Rossing K, Christensen PK, Hovind P, et al. Progression of nephropathy in type 2 diabetic patients. *Kidney Int* 2004;66:1596-1605.
11. Mehdi U, Toto RD. Anemia, diabetes, and chronic kidney disease. *Diabetes Care* 2009;32:1320-1326.
12. Hara A, Furuichi K, Higuchi M, et al. Autoantibodies to erythropoietin receptor in patients with immune-mediated diseases: relationship to anaemia with erythroid hypoplasia. *Br J Haematol* 2013;160:244-250.
13. Hara A, Furuichi K, Yamahana J, et al. Effect of Autoantibodies to Erythropoietin Receptor in Systemic Lupus Erythematosus with Biopsy-proven Lupus Nephritis. *J*

Rheumatol 2016;43:1328-1334.

14. Matsuo S, Imai E, Horio M, et al. Collaborators developing the Japanese equation for estimated GFR: Revised equations for estimated GFR from serum creatinine in Japan. *Am J Kidney Dis* 2009;53:982-992.
15. Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus, Seino Y, Nanjo K, Tajima N, et al. Report of the committee on the classification and diagnostic criteria of diabetes mellitus. *J Diabetes Investig* 2010;1:212-228.
16. Tervaert TW, Mooyaart AL, Amann K, et al.; Renal Pathology Society: Pathologic classification of diabetic nephropathy. *J Am Soc Nephrol* 2010;21:556-563.
17. Anagnostou A, Liu Z, Steiner M, et al. Erythropoietin receptor mRNA expression in human endothelial cells. *Proc Natl Acad Sci U S A* 1994;91:3974-3978.
18. Miyazaki Y, Kuriyama K, Higuchi M, et al. Establishment and characterization of a new erythropoietin-dependent acute myeloid leukemia cell line, AS-E2. *Leukemia* 1997;11:1941-1949.
19. Schröppel B, Fischereder M, Wiese P, et al. Expression of glucose transporters in human peritoneal mesothelial cells. *Kidney Int* 1998;53:1278-1287.

20. Chang DC, Piaggi P, Hanson RL, et al. Use of a High-Density Protein Microarray to Identify Autoantibodies in Subjects with Type 2 Diabetes Mellitus and an HLA Background Associated with Reduced Insulin Secretion. *PLoS One* 2015;10:e0143551.

21. Ashraf JM, Arfat MY, Arif Z, et al. A clinical correlation of anti-DNA-AGE autoantibodies in type 2 diabetes mellitus with disease duration. *Cell Immunol* 2015;293:74-79.

22. El-Lebedy D, Rasheed E, Kafoury M, et al. Anti-apolipoprotein A-1 autoantibodies as risk biomarker for cardiovascular diseases in type 2 diabetes mellitus. *J Diabetes Complications* 2016;30:580-585.

23. Fredrikson GN, Anand DV, Hopkins D, et al. Associations between autoantibodies against apolipoprotein B-100 peptides and vascular complications in patients with type 2 diabetes. *Diabetologia* 2009;52:1426-1433.

24. Wada T, Furuichi K, Sakai N, et al. Up-regulation of monocyte chemoattractant protein-1 in tubulointerstitial lesions of human diabetic nephropathy. *Kidney Int* 2000;58:1492-1499.

25. Zeisberg M, Neilson EG. Mechanisms of tubulointerstitial fibrosis. *J Am Soc Nephrol* 2010;21:1819-1834.

26. Cravedi P, Manrique J, Hanlon KE, et al. Immunosuppressive effects of erythropoietin on human alloreactive T cells. *J Am Soc Nephrol* 2014;25:2003-2015.

27. Lisowska KA, Debska-Slizień A, Bryl E, et al. Erythropoietin receptor is expressed on human peripheral blood T and B lymphocytes and monocytes and is modulated by recombinant human erythropoietin treatment. *Artif Organs* 2010;34:654-662.

28. De Beuf A, Hou XH, D'Haese PC, et al. Epoetin delta reduces oxidative stress in primary human renal tubular cells. *J Biomed Biotechnol* 2010;2010:395785.

29. Beleslin-Cokic BB, Cokic VP, Yu X, et al. Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells. *Blood* 2004;104:2073-2080.

30. Pearl RG. Erythropoietin and organ protection: lessons from negative clinical trials. *Crit Care* 2014;18:526.

31. Kuriyama S, Tomonari H, Yoshida H, et al. Reversal of anemia by erythropoietin therapy retards the progression of chronic renal failure, especially in nondiabetic patients. *Nephron* 1997;77:176-185.

32. Tsubakihara Y, Gejyo F, Nishi S, et al. High target hemoglobin with erythropoiesis-stimulating agents has advantages in the renal function of

non-dialysis chronic kidney disease patients. *Ther Apher Dial* 2012;16:529-540.

33. Cody J, Daly C, Campbell M, et al. Recombinant human erythropoietin versus placebo or no treatment for the anaemia of chronic kidney disease in people not requiring dialysis. *Cochrane Database Syst Rev* 2016;1:CD003266.

Figure legends

Figure 1. Renal outcome of the retrospective analysis of 112 type 2 diabetic patients with chronic kidney disease.

Event-free rate of end-stage renal disease (ESRD) stratified by the presence or absence of autoantibodies to the erythropoietin receptor (EPOR) according to the Kaplan-Meier method. Dotted line, anti-EPOR-negative (n = 86); solid line, anti-EPOR-positive (n = 26). The mean follow-up period was 45.3 ± 47.4 months. Differences between the groups were compared using a log-rank test.

Figure 2. Effect of EPO and IgG fractions with anti-EPOR antibodies on the expression of MCP-1 mRNA in HK-2 cells.

(A) EPOR mRNA was assessed by RT-PCR. 5mMG: 5mM D-glucose, 20mMG: 20mM D-glucose, hEPOR: human erythropoietin receptor (B) Stimulation of HK-2 with 20mM D-glucose increased the expression of MCP-1 mRNA, and EPO decreased it (n = 3). 5mM Glu: 5mM D-glucose, 20mM Glu: 20mM D-glucose, EPO: erythropoietin (C) Pretreatment with the IgG fraction from a patient with anti-EPOR antibodies under 20mM glucose upregulate the expression of MCP-1 mRNA (n =3). 20mM Glu: 20mM

D-glucose, EPO: erythropoietin, a-EPOR Ab: anti-erythropoietin receptor antibodies.

All values are the mean ± SD. *P < 0.05.

Supplementary Figure 1. Renal outcome of type 2 diabetic patients according to baseline eGFR levels below and above 30 mL/min/1.73m^2

Event-free rate of ESRD stratified by the presence or absence of autoantibodies to the EPOR according to the Kaplan-Meier method. (A) Patients with baseline eGFR below 30 mL/min/1.73m^2. Dotted line, anti-EPOR-negative (n = 30); solid line, anti-EPOR-positive (n = 17). (B) Patients with baseline eGFR above 30 mL/min/1.73m^2. Dotted line, anti-EPOR-negative (n = 56); solid line, anti-EPOR-positive (n = 9). Differences between the groups were compared using a log-rank test.

Supplementary Figure 2. Renal outcome of type 2 diabetic patients according to baseline eGFR levels below and above 60 mL/min/1.73m^2

Event-free rate of ESRD stratified by the presence or absence of autoantibodies to the EPOR according to the Kaplan-Meier method. (A) Patients with baseline eGFR below 60 mL/min/1.73m^2. Dotted line, anti-EPOR-negative (n = 60); solid line, anti-EPOR-positive (n = 21). (B) Patients with baseline eGFR above 60 mL/min/1.73m^2. Dotted line, anti-EPOR-negative (n = 26); solid line, anti-EPOR-positive (n = 5). Differences between the groups were compared using a log-rank test.

Table 1. Baseline clinical parameters for all patients and those with and without anti-EPOR antibodies.

Clinical parameters	All patients (n=112)	Anti-EPOR-negative (n=86)	Anti-EPOR-positive (n=26)	*P*-value
Male	76	58	18	0.86
Age (years)	62.9 ± 12.5	61.5 ± 12.3	67.5 ± 12.2	<0.05
Proteinuria (g/day)	2.7 ± 3.1	2.7 ± 3.1	2.8 ± 3.1	0.9
Serum creatinine（mg/dL）	2.4 ± 2.2	2.1 ± 2.0	3.1 ± 2.4	<0.05
eGFR（mL/min/1.73m^2）	42.0 ± 30.2	45.4 ± 30.2	30.7 ± 27.7	<0.05
Duration of diabetes (years)	13.6 ± 9.8	13.1 ± 9.9	15.0 ± 9.7	0.41
HbA1c (%)	6.7 ± 1.5	6.8 ± 1.6	6.4 ± 1.3	0.22
BMI (kg/m^2)	24.3 ± 4.0	24.7 ± 4.1	22.6 ± 3.3	<0.05
SBP (mmHg)	136 ± 24	135 ± 23	137 ± 27	0.75
DBP (mmHg)	74 ± 14	75 ± 14	72 ± 14	0.42
T-Chol (mg/dL)	180 ± 51	186 ± 50	160 ± 47	<0.05
HDL-C (mg/dL)	45 ± 18	47 ± 20	40 ± 12	0.12
Triglyceride (mg/dL)	142 ± 79	152 ± 85	108 ± 39	<0.01
Hemoglobin (g/dL)	11.2 ± 2.0	11.5 ± 2.0	10.1 ± 1.7	<0.01
Reticulocytes（×10^4/μL）[a]	4.4 ± 2.2	4.4 ± 2.0	4.3 ± 2.7	0.96
Fe (mg/dL)	71.2 ± 40.6	73.3 ± 43.2	66.3 ± 34.0	0.49
TIBC (mg/dL)	244 ± 60	246 ± 65	239 ± 47	0.62
Ferritin (ng/mL)	233 ± 226	227 ± 206	248 ± 274	0.71
CRP (mg/dL)	0.6 ± 1.3	0.5 ± 1.2	0.9 ± 1.7	0.22
Use of ACE inhibitors or ARBs (%)	81	81	79	0.82
Use of ESA (%)	30	19	64	<0.01

*n = 73

eGFR: estimated glomerular filtration rate; BMI: body mass index; SBP: systolic blood

pressure; DBP: diastolic blood pressure; T-Chol: total cholesterol; HDL-C: HDL cholesterol; TIBC: total iron binding capacity; CRP: C-reactive protein; ACE: angiotensin-converting enzyme; ARB: angiotensin II receptor blocker; ESA: erythropoiesis stimulating agent

Table 2. Pathological features of patients with and without anti-EPOR antibodies.

Pathological parameters	Anti-EPOR-negative (n=41)	Anti-EPOR-positive (n=10)	*P*-value
Diffuse lesions scale 3 or 4	24 (58.5)	3 (30.0)	0.16
Nodular lesion	17 (41.5)	3 (30.0)	0.72
Exudative lesion	17 (41.5)	3 (30.0)	0.72
Mesangiolysis	16 (39.0)	3 (30.0)	0.73
IFTA score 3	13 (31.7)	3 (30.0)	1
Interstitial inflammation			0.03
score 0	5 (12.2)	0 (0.0)	
score 1	28 (68.3)	4 (40.0)	
score 2	8 (19.5)	6 (60.0)	
Arteriolar hyalinosis grade 2 or 3	8 (19.5)	5 (50.0)	0.10
Arteriosclerosis score 2*	6 (17.6)	1 (16.7)	1

*n = 34 in the anti-EPOR-negative group, n = 6 in the anti-EPOR-positive group

EPOR: erythropoietin receptor; IFTA: interstitial fibrosis and tubular atrophy

Table 3. Parameters identified by multivariate Cox proportional hazards regression analysis associated with ESRD.

	HR	95% CI	*P*-value
Age per -1 year	1.04	1.01-1.07	<0.05
SBP per 1mmHg	1.04	1.02-1.06	<0.01
Proteinuria per 1g/day	1.18	1.01-1.38	<0.05
eGFR per -1 mL/min/1.73m^2	1.05	1.02-1.07	<0.01
Presence of anti-EPOR antibodies	2.78	1.20-6.43	<0.05

SBP: systolic blood pressure; eGFR: estimated glomerular filtration rate; EPOR: erythropoietin receptor; HR: hazard ratio; CI: confidence interval

HRs are adjusted for sex, age, body mass index, SBP, proteinuria, eGFR, HbA1c, hemoglobin, and presence of anti-EPOR antibodies

Supplementary Table 1. Baseline characteristics of patients according to baseline eGFR levels below and above 30 mL/min/1.73m^2

Clinical parameters	eGFR < 30 mL/min/1.73m^2		eGFR ≥ 30 mL/min/1.73m^2	
	Anti-EPOR-negative (n=30)	Anti-EPOR-positive (n=17)	Anti-EPOR-negative (n=56)	Anti-EPOR-positive (n=9)
Male	20	13	38	5
Age (years)	64.2 ± 12.8	67.8 ± 12.0	60.1 ± 11.9	66.9 ± 13.1
Proteinuria (g/day)	4.3 ± 3.9	3.4 ± 3.6	1.7 ± 2.0	1.6 ± 1.4
Serum creatinine（mg/dL）	4.1 ± 2.3	4.3 ± 2.3	1.1 ± 0.4	1.0 ± 0.4
eGFR（mL/min/1.73m^2）	15.4 ± 7.8	15.0 ± 8.4	61.5 ± 24.9	60.2 ± 27.5
Duration of diabetes (years)	16.6 ± 11.8	14.7 ± 10.4	11.3 ± 8.3	15.7 ± 8.2
HbA1c (%)	6.3 ± 1.1	6.2 ± 1.4	7.1 ± 1.7	6.8 ± 1.1
BMI (kg/m^2)	24.9 ± 4.3	22.2 ± 2.6*	24.7 ± 4.1	23.4 ± 4.3
SBP (mmHg)	137 ± 29	138 ± 30	134 ± 19	135 ± 19
DBP (mmHg)	72 ± 17	70 ± 16	76 ± 12	76 ± 12
T-Chol (mg/dL)	183 ± 56	153 ± 52	188 ± 47	171 ± 35
HDL-C (mg/dL)	47 ± 26	36 ± 10	47 ± 15	47 ± 11
Triglyceride (mg/dL)	160 ± 99	106 ± 42	148 ± 77	112 ± 35
Hemoglobin (g/dL)	10.4 ± 1.7	9.5 ± 0.9*	12.1 ± 1.9	11.2 ± 2.2
Reticulocytes（×10^4/μL）	4.5 ± 2.1	4.8 ± 2.9	4.3 ± 2.0	3.2 ± 2.1
Fe (mg/dL)	59 ± 35	61 ± 28	87 ± 46	78 ± 45
TIBC (mg/dL)	221 ± 70	230 ± 50	271 ± 49	260 ± 35
Ferritin (ng/mL)	249 ± 200	258 ± 320	207 ± 213	227 ± 150
CRP (mg/dL)	0.65 ± 1.20	1.07 ± 1.72	0.53 ± 1.29	0.79 ± 1.87
Use of ACE inhibitors or ARBs (%)	71	81	87	75
Use of ESA (%)	45	94**	6	11

eGFR: estimated glomerular filtration rate; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; T-Chol: total cholesterol; HDL-C: HDL cholesterol; TIBC: total iron binding capacity; CRP: C-reactive protein; ACE: angiotensin-converting enzyme; ARB: angiotensin II receptor blocker; ESA: erythropoiesis stimulating agent

* $p<0.05$, ** $p<0.01$

Supplementary Table 2. Baseline characteristics of patients according to baseline eGFR levels below and above 60 mL/min/1.73m^2

Clinical parameters	eGFR < 60 mL/min/1.73m^2		eGFR ≥ 60 mL/min/1.73m^2	
	Anti-EPOR-negative (n=60)	Anti-EPOR-positive (n=21)	Anti-EPOR-negative (n=26)	Anti-EPOR-positive (n=5)
Male	43	15	15	3
Age (years)	61.6 ± 12.4	67.9 ± 12.0*	61.3 ± 12.3	65.6 ± 14.0
Proteinuria (g/day)	3.1 ± 3.3	3.1 ± 3.3	1.6 ± 2.2	1.3 ± 1.3
Serum creatinine（mg/dL）	2.7 ± 2.2	3.7 ± 2.3	0.7 ± 0.2	0.7 ± 0.2
eGFR（mL/min/1.73m^2）	29.3 ± 16.7	19.0 ± 11.3*	82.6 ± 19.5	80.0 ± 20.2
Duration of diabetes (years)	14.6 ± 10.5	15.3 ± 9.7	9.8 ± 7.2	13.0 ± 11.4
HbA1c (%)	6.7 ± 1.5	6.4 ± 1.5	7.1 ± 1.8	6.3 ± 0.5
BMI (kg/m^2)	24.9 ± 4.2	22.4 ± 2.3*	24.4 ± 4.1	23.8 ± 6.0
SBP (mmHg)	136 ± 25	136 ± 28	133 ± 16	143 ± 21
DBP (mmHg)	74 ± 15	71 ± 15	76 ± 9	79 ± 10
T-Chol (mg/dL)	183 ± 54	152 ± 48*	194 ± 42	186 ± 35
HDL-C (mg/dL)	46 ± 21	40 ± 13	50 ± 17	42 ± 5
Triglyceride (mg/dL)	160 ± 93	104 ± 40*	135 ± 63	121 ± 34
Hemoglobin (g/dL)	10.9 ± 1.8	9.8 ± 1.2**	12.7 ± 1.9	11.2 ± 2.7
Reticulocytes（×10^4/μL）	4.5 ± 2.2	4.8 ± 2.7	4.0 ± 1.4	2.2 ± 1.6
Fe (mg/dL)	69 ± 35	66 ± 36	93 ± 68	66 ± 25
TIBC (mg/dL)	244 ± 70	234 ± 50	254 ± 34	261 ± 31
Ferritin (ng/mL)	212 ± 174	235 ± 300	288 ± 308	307 ± 105
CRP (mg/dL)	0.56 ± 1.25	0.91 ± 1.60	0.60 ± 1.30	1.20 ± 2.35
Use of ACE inhibitors or ARBs (%)	80	84	84	60
Use of ESA (%)	26	80**	4	0

eGFR: estimated glomerular filtration rate; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; T-Chol: total cholesterol; HDL-C: HDL cholesterol; TIBC: total iron binding capacity; CRP: C-reactive protein; ACE: angiotensin-converting

enzyme; ARB: angiotensin II receptor blocker; ESA: erythropoiesis stimulating agent

* $p<0.05$, ** $p<0.01$