ORIGINAL ARTICLE

Early growth response 1 as a podocyte injury marker in human glomerular diseases

Masahiro Okabe [1], Kentaro Koike[1], Izumi Yamamoto [1], Nobuo Tsuboi [1], Taiji Matsusaka[2] and Takashi Yokoo[1]

[1]Division of Nephrology and Hypertension, Department of Internal Medicine, Jikei University School of Medicine, Tokyo, Japan and [2]Departments of Basic Medicine, Tokai University School of Medicine, Isehara, Japan

Correspondence to: Masahiro Okabe; E-mail: mokabe@jikei.ac.jp

ABSTRACT

Background. In human glomerular diseases, visualizing podocyte injury is desirable since podocytes do not regenerate and podocyte injury leads to podocyte loss. Herein, we investigated the utility of immunostaining for early growth response 1 (EGR1), which is expressed in injured podocytes from the early stages of injury in animal experiments, as a podocyte injury marker in human glomerular diseases.

Methods. This study included 102 patients with biopsy-proven glomerular diseases between 2018 and 2021. The proportion of EGR1 expression in podocytes (%EGR1pod) was analyzed in relation to clinical and histopathological features, including glomerular and urinary podocyte-specific markers.
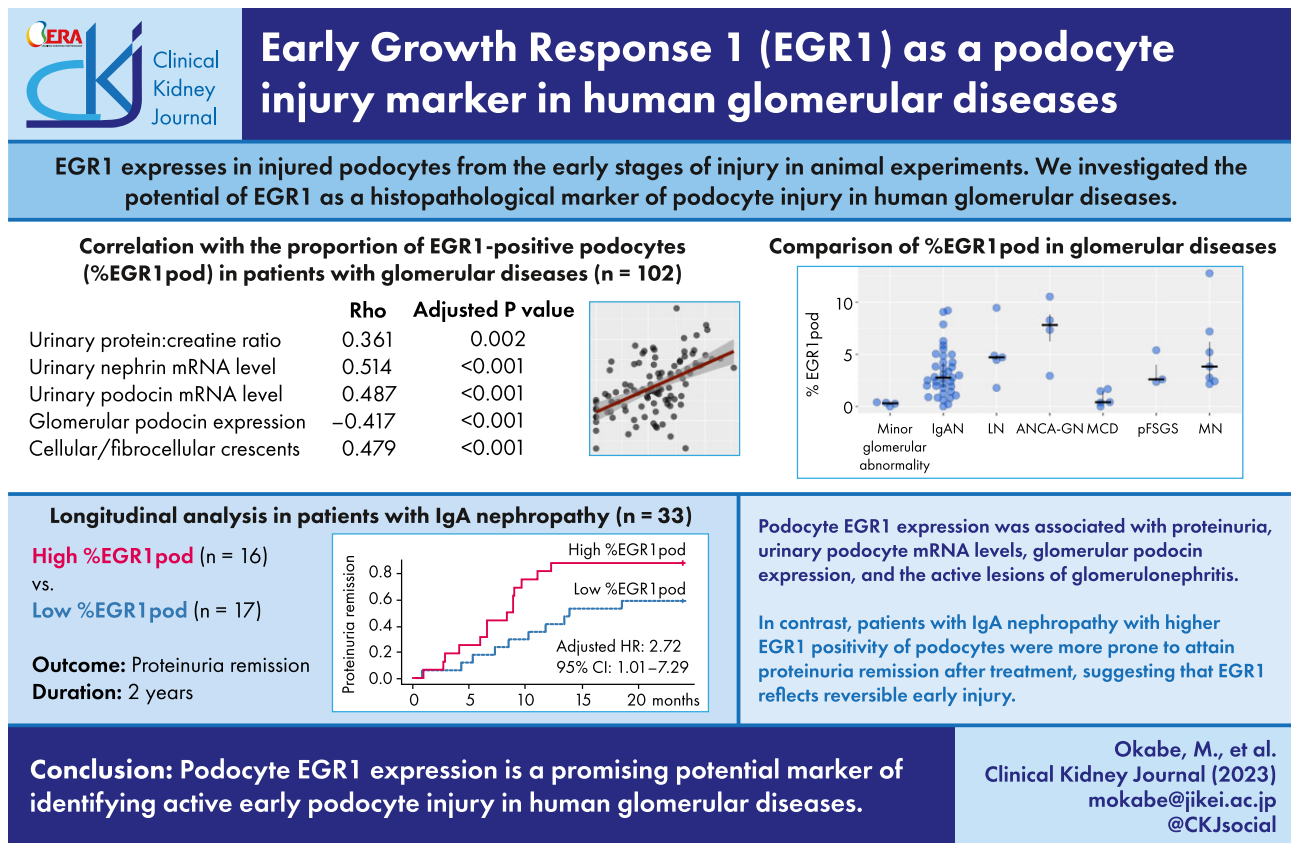
Results. %EGR1pod correlated significantly with the urinary protein:creatinine ratio, urinary nephrin and podocin mRNA levels, and glomerular podocin staining (rho = 0.361, 0.514, 0.487 and –0.417, respectively; adjusted P = .002, <.001, <.001 and <.001, respectively). Additionally, %EGR1pod correlated with cellular/fibrocellular crescents (rho = 0.479, adjusted P <.001). %EGR1pod was high in patients with glomerulonephritis, such as immunoglobulin A nephropathy (IgAN), lupus nephritis and antineutrophil cytoplasmic antibody–associated glomerulonephritis, and in those with podocytopathies, such as membranous nephropathy and primary focal segmental glomerulosclerosis, while %EGR1pod was low in patients with minimal change disease. In a subgroup analysis of IgAN, %EGR1pod was higher in Oxford C1 patients than in C0 patients. However, unexpectedly, patients with higher %EGR1pod were more prone to attain proteinuria remission, suggesting that EGR1 in the context of IgAN reflects reversible early injury.

Conclusions. Our findings indicate that EGR1 is a promising potential marker for identifying active early podocyte injury in human glomerular diseases.

GRAPHICAL ABSTRACT

Clinical Kidney Journal

Early Growth Response 1 (EGR1) as a podocyte injury marker in human glomerular diseases

EGR1 expresses in injured podocytes from the early stages of injury in animal experiments. We investigated the potential of EGR1 as a histopathological marker of podocyte injury in human glomerular diseases.

Correlation with the proportion of EGR1-positive podocytes (%EGR1pod) in patients with glomerular diseases (n = 102)

	Rho	Adjusted P value
Urinary protein:creatine ratio	0.361	0.002
Urinary nephrin mRNA level	0.514	<0.001
Urinary podocin mRNA level	0.487	<0.001
Glomerular podocin expression	−0.417	<0.001
Cellular/fibrocellular crescents	0.479	<0.001

Comparison of %EGR1pod in glomerular diseases

Longitudinal analysis in patients with IgA nephropathy (n = 33)

High %EGR1pod (n = 16)
vs.
Low %EGR1pod (n = 17)

Outcome: Proteinuria remission
Duration: 2 years

Adjusted HR: 2.72
95% CI: 1.01–7.29

Podocyte EGR1 expression was associated with proteinuria, urinary podocyte mRNA levels, glomerular podocin expression, and the active lesions of glomerulonephritis.

In contrast, patients with IgA nephropathy with higher EGR1 positivity of podocytes were more prone to attain proteinuria remission after treatment, suggesting that EGR1 reflects reversible early injury.

Conclusion: Podocyte EGR1 expression is a promising potential marker of identifying active early podocyte injury in human glomerular diseases.

Okabe, M., et al.
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mokabe@jikei.ac.jp
@CKJsocial

KEY LEARNING POINTS

What was known:

- Podocyte injury is associated with the initiation and progression of glomerular diseases because podocytes rarely proliferate or regenerate.
- There is no established marker that is enhanced in injured podocytes in human glomerular diseases.
- Early growth response 1 (EGR1) is expressed in injured podocytes from the early stages of injury in animal experiments.

This study adds:

- EGR1 expression in podocytes was significantly positively correlated with proteinuria, urinary podocyte mRNA levels and cellular/fibrocellular crescents, and was significantly negatively correlated with glomerular podocin staining in various human glomerular diseases.
- In the early phase of immunoglobulin A nephropathy, patients with higher EGR1 positivity of podocytes were more prone to recovery, suggesting that EGR1 reflects reversible early injury.
- EGR1 could be a promising potential marker for identifying active early podocyte injury in human glomerular diseases.

Potential impact:

- Identifying EGR1-positive podocytes coupled with intensive treatment could potentially improve the kidney prognosis of patients with glomerular diseases.

INTRODUCTION

Podocytes are terminally differentiated, highly specialized epithelial cells that cover the outer surfaces of glomerular capillaries. Accumulating evidence has shown the importance of podocyte injury in the initiation and progression of glomerular diseases. Podocytes exposed to severe and/or persistent injuries detach from the glomerulus into the urine; substantial podocyte loss leads to glomerulosclerosis because podocytes

rarely proliferate or regenerate [1–3]. A decrease in nephron number increases intraglomerular pressure on the residual glomeruli, leading to the progression of kidney diseases. More-over, podocyte injury can spread to other podocytes in the glomerulus [4–6]. Thus, the early control of podocyte injury is crucial to inhibit the progression of glomerular diseases.

Proteinuria and albuminuria tests are frequently used for podocyte injury detection but are not specific to podocyte injury. Therefore, as more specific biomarkers for podocyte injury, quantitative approaches for urinary podocyte number, urinary podocyte proteins, urinary podocyte mRNAs, urinary exosomes and urinary microparticles have been reported [7–17]. Although such biomarkers are useful because they can be noninva-sively and repeatably measured, visualizing injuries of resi-dent podocytes in tissues prior to their detachment from the glomerulus is essential. Therefore, immunostaining analysis of podocyte-associated proteins, such as nephrin, podocin and podocalyxin, is performed. However, decreased expression of these proteins cannot distinguish podocyte injury from deple-tion. Thus, a biomarker expressed by injured podocytes in hu-man glomerular diseases, such as desmin in rodents, is desirable to detect podocyte injury.

Early growth response 1 (EGR1) is a zinc finger transcrip-tion factor that regulates cell survival, proliferation, cell death and fibrosis in response to DNA damage and ischemia [18, 19]. We detected a high expression of *Egr1* in injured podocytes of NEP25 mice, an inducible podocyte injury model [3, 20]. *Egr1* mRNA levels have been shown to increase 70-fold on day 4 after the induction of podocyte injury, when the expression of podocyte-associated proteins was not yet decreased. EGR1-positive podocytes expressed desmin. Using podocyte-selective *Tln1* conditional knockout mice, another group reported that EGR1 was expressed in podocytes shortly after the induction of *Tln1* knockout and that *Egr1* knockout ameliorated albuminuria and glomerulosclerosis [21]. *EGR1* is conserved across species; the EGR1 protein has been reported to be expressed in podocytes in human glomerular diseases, such as membranous nephropa-thy (MN), immunoglobulin A nephropathy (IgAN) and focal seg-mental glomerulosclerosis (FSGS), and *EGR1* mRNA is expressed in diabetic nephropathy [20–22]. However, EGR1 expression at the mRNA and protein levels was rarely observed in podocytes of intact glomeruli [20, 21, 23].

In this study, we aimed to explore the clinical utility of EGR1 staining as a podocyte injury marker in human glomerular dis-eases. We discovered that EGR1 expression in podocytes cor-related significantly with proteinuria, urinary podocyte mRNA levels and cellular/fibrocellular crescents, and inversely with glomerular podocin expression. EGR1 expression was observed in the podocytes of patients with various glomerular diseases but was less frequent in patients with minimal change disease (MCD). Moreover, EGR1-positive podocytes can be recovered in IgAN since patients with higher EGR1 positivity of podocytes were more prone to attain proteinuria remission after intensive treatment.

MATERIALS AND METHODS

Patients and ethics

This single-center cross-sectional and prospective study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Jikei University School of Medicine [approval number 30-048(9069)]. Written informed consent was obtained from all participants included in this study.

Patients with primary and secondary glomerular diseases who underwent kidney biopsies at the Jikei University Hospital, Tokyo, from June 2018 to December 2021 were recruited. The following patients were excluded: patients under 20 years of age, patients with tubulointerstitial diseases and kidney transplant patients. Patients with minor glomerular abnormal-ities (MGA) with mild proteinuria and/or microhematuria were included.

Clinical data and laboratory data, including serum creati-nine level, urinary protein:creatinine ratio (UPCR), 24-h urinary protein excretion (UPE) level, 24-h creatinine clearance (CCr) level and hematuria, were obtained from the patient's medi-cal records at the time of diagnostic kidney biopsy. The esti-mated glomerular filtration rate (eGFR) was calculated using a creatinine-based equation for the Japanese population [24].

Histopathological evaluation

Histopathological data, including pathological diagnosis, the total number of glomeruli obtained from biopsy specimens, and glomeruli containing global or segmental glomeruloscle-rosis, endocapillary hypercellularity and crescents, were ob-tained from reports by pathologists and reviewed by the authors. The degree of tubulointerstitial damage was semi-quantitatively evaluated as increased extracellular matrix separating tubules and atrophic tubules and expressed as a percentage of the af-fected area over the observed cortical area, with 1%–5% rounded to 5%, and other values rounded to the nearest 10%. The Oxford classification was used to evaluate the histopathological find-ings of IgAN patients [25].

Paraffin sections (3-µm thick) of kidney biopsies were immunostained with antibodies against EGR1, podocin and Wilms' tumor 1 (WT1) (antibody list can be found in Supplementary data, Table S1). Sections subjected to EGR1 im-munostaining were counterstained with a periodic acid–Schiff (PAS) stain. EGR1-positive cells located along the outside of the glomerular basement membrane (GBM) were identified as EGR1-positive podocytes using serial sections stained for WT1 and podocin. The total number of EGR1-positive podocytes per total number of WT1-positive podocytes in the kidney section of each patient was designated as %EGR1pod. The percentage of glomeruli, including EGR1-positive cells located along the outside of the GBM among non-globally sclerotic glomeruli, was designated as %EGR1glo. The glomerular podocin score was calculated by averaging the scores of all non-globally sclerotic glomeruli assigned on a scale of 0–8 according to the degree of podocin immunostaining in a paraffin section (Supplementary data, Fig. S1) [5].

The minimum number of non-globally sclerotic glomeruli evaluated per section was set to eight. Small (diameter <50 µm) and partial glomeruli were excluded from the immunostain-ing evaluation. Since it is difficult to identify podocytes in the vicinity of the crescent, glomeruli with full-moon cres-cents were excluded from the immunostaining analysis. Fur-thermore, in glomeruli with segmental crescents, only EGR1-positive podocytes located away from the crescent were counted (Supplementary data, Fig. S2).

Urine samples

The procedures for urine sampling and measurements were conducted according to the method described by Fukuda et al.,
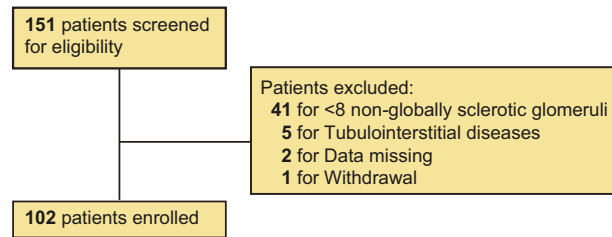
Figure 1: Flowchart of patient enrollment.

with minor modifications [15]. Briefly, urine samples were collected in the morning during the kidney biopsy and centrifuged; the pellet was used for the mRNA measurement, and the supernatant was used for the podocalyxin protein measurement. Quantitation of nephrin (TaqMan probes, Thermo Fisher Scientific, Waltham, MA, USA: Hs00190446_m1) and podocin (Hs00922492_m1) mRNA abundance was performed using a Rotor-Gene Q real-time PCR cycler (Qiagen, Venlo, The Netherlands). Standard curves were constructed using serially diluted standards comprising cDNA reverse-transcribed from human kidney total RNA (Clontech Laboratories, Mountain View, CA, USA). The quantity of nephrin or podocin mRNA in 10 μL of the 1:10 000 diluted standard was set as 1 U. Podocalyxin protein levels were measured using a sandwich enzyme-linked immunosorbent assay kit for human podocalyxin (Aviva Systems Biology OKEH02869, San Diego, CA, USA). The concentrations of urinary nephrin mRNA, podocin mRNA and podocalyxin protein were standardized to the urinary creatinine concentration. The precise methods are described in the Supplementary Methods.

Statistical analysis

The clinical and pathological variables of the patients are presented as medians and interquartile ranges or numbers and percentages. Correlations between variables were assessed using Spearman's rank correlation. Differences were evaluated using the Mann–Whitney U test for two groups and the Kruskal–Wallis test for more than three groups. The Bonferroni–Holm method or Steel's method was used for multiple comparison corrections. The Kaplan–Meier curve was applied to assess the prognostic value of podocyte EGR1 expression for proteinuria remission, defined as at least two consecutive findings of <0.3 g/g of UPCR, in IgAN patients. Hazard ratio (HR) and 95% confidence interval (95% CI), adjusted by age, sex, baseline UPCR and eGFR, and corticosteroid use, were calculated using the Cox proportional hazards model. Statistical significance was defined as a two-sided P-value <.05. All statistical analyses were performed using R software version 4.3.1.

RESULTS

Baseline characteristics of the patients

In total, 102 patients were enrolled in this study (Fig. 1). All patients underwent a single kidney biopsy during the study period. Table 1 presents the clinical and histopathological characteristics of the included patients. All participants were Japanese, and the median %EGR1pod was 2.45 (1.11–4.65)%. Figure 2 shows the representative immunostaining for EGR1 with WT1 and podocin in serial sections.

Correlation between podocyte EGR1 expression and clinical parameters

%EGR1pod correlated significantly with UPCR, UPE level, and urinary nephrin and podocin mRNA:creatinine ratios (rho = 0.361, 0.342, 0.514 and 0.487, respectively; adjusted P = .002, .006, <.001 and <.001, respectively) (Table 2, Fig. 3, Supplementary data, Fig. S3). %EGR1pod correlated weakly with urinary podocalyxin levels without statistical significance (rho = 0.253); %EGR1pod did not correlate with eGFR, 24-h CCr or hematuria.

Podocyte EGR1 expression and kidney biopsy histopathological features

%EGR1pod was significantly and inversely correlated with glomerular podocin score (rho = −0.417, adjusted P <.001) but did not show a significant correlation with the podocyte number (Table 2, Fig. 3, Supplementary data, Fig. S3). Podocyte EGR1 expression was significantly associated with cellular/fibrocellular crescents (rho = 0.479, adjusted P <.001) and tended to be weakly associated with tubulointerstitial damage (rho = 0.272, adjusted P = .051) but not with global glomerulosclerosis or fibrous crescents. EGR1-positive binucleated podocytes were occasionally observed (Supplementary data, Fig. S4).

Comparison of podocyte EGR1 expression among glomerular diseases

Podocyte EGR1 expression was also compared among representative glomerular diseases. Compared with MGA [0.31 (0.20–0.37)%], %EGR1pod was significantly higher in IgAN and MN [2.76 (1.77–4.23)% and 3.83 (2.59–6.19)%, respectively; adjusted P = .015 and .037, respectively] (Fig. 4). The %EGR1pod of lupus nephritis (LN), antineutrophil cytoplasmic antibody–associated glomerulonephritis (ANCA-GN) and primary FSGS tended to be higher than that of MGA [4.71 (4.46–4.88)%, 7.81 (6.24–8.84)% and 2.60 (2.48–4.00)%, respectively; adjusted P = .063, .088 and .135, respectively]. In contrast, MCD had a low %EGR1pod [0.42 (0.38–1.47)%].

Subgroup analyses of patients with IgAN

Next, we performed a subgroup analysis of the 37 patients with IgAN (Supplementary data, Table S2). In this population, %EGR1pod correlated significantly with UPE level (rho = 0.559, adjusted P = .011) (Table 3, Supplementary data, Fig. S5). UPCR, urinary nephrin and podocin mRNA levels, urinary podocalyxin protein level, glomerular podocin scores and endocapillary hypercellularity correlated moderately with %EGR1pod, with no statistical significance in this subgroup (rho = 0.432, 0.375, 0.4, 0.417, −0.362 and 0.403; adjusted P = .114, .184, .162, .139, .279 and .161, respectively). %EGR1pod tended to be associated with cellular/fibrocellular crescents (rho = 0.442, adjusted P = .092), and patients with Oxford C1 lesions had a significantly higher %EGR1pod than those with C0 lesions (adjusted P = .030) (Fig. 5).

The 33 patients with IgAN, who had UPCR of 0.3 g/g or more at kidney biopsy and were followed for >1 year, were divided into two groups by the median of %EGR1pod (2.99%) and longitudinally observed over a period of 2 years (Supplementary data, Table S3). Proteinuria remission was observed more frequently in the higher %EGR1pod group [adjusted HR 2.72 (95% CI 1.01–7.29)] (Fig. 6A, Supplementary data, Table S4). The subgroup analysis by the podocyte number showed that the group with a

Table 1: Patient characteristics ($n = 102$).

Characteristic	Value
Clinical findings	
Age, years	49 (36–59)
Sex, no. (%)	
Female	43 (42.2)
Male	59 (57.8)
Height, cm	164 (159–171)
Weight, kg	62.6 (55.2–73.2)
BMI, kg/m^2	23.0 (20.9–25.3)
Hypertension, no. (%)	51 (50.0)
RAAS inhibitors usage, no. (%)	40 (39.2)
Diabetes mellitus, no. (%)	13 (12.7)
Laboratory findings	
Serum creatinine level, mg/dL	0.93 (0.73–1.19)
eGFR, mL/min/1.73 m^2	65.0 (43.5–79.0)
24-h CCr, mL/min[a]	86.0 (63.5–112.0)
UPCR, g/g	0.88 (0.53–2.40)
UPE, g/day[b]	0.96 (0.50–2.55)
Urinary RBC count, no. (%)	
Grade 0, 0–4 cells/HPF	33 (32.4)
Grade 1, 5–9 cells/HPF	9 (8.8)
Grade 2, 10–19 cells/HPF	16 (15.7)
Grade 3, 20–49 cells/HPF	24 (23.5)
Grade 4, 50–99 cells/HPF	10 (9.8)
Grade 5, ≥100 cells/HPF	10 (9.8)
Urinary nephrin mRNA:creatinine ratio, ×10^4 U/g	5.33 (2.35–23.26)
Urinary podocin mRNA:creatinine ratio, ×10^4 U/g	1.74 (0.56–7.22)
Urinary podocalyxin:creatinine ratio, ×10^{-5} g/g	1.49 (0.69–2.80)
Diagnosis	
IgAN, no. (%)	37 (36.3)
MN, no. (%)	7 (6.9)
LN, no. (%)	5 (4.9)
MCD, no. (%)	5 (4.9)
ANCA-GN, no. (%)	4 (3.9)
Primary FSGS, no. (%)	3 (2.9)
IgA vasculitis with nephritis, no. (%)	3 (2.9)
Obesity-related glomerulopathy, no. (%)	3 (2.9)
Thin basement membrane disease, no (%)	2 (2.0)
Primary MPGN, no (%)	2 (2.0)
Diabetic nephropathy, no (%)	1 (1.0)
Poststreptococcal glomerulonephritis, no (%)	1 (1.0)
Light-chain deposition disease, no (%)	1 (1.0)
PGNMID, no (%)	1 (1.0)
Hypertensive nephrosclerosis, no. (%)	10 (9.8)
Secondary FSGS, no. (%)	5 (4.9)
Minor glomerular abnormalities, no. (%)	4 (3.9)
Others, no. (%)	8 (7.8)
Histopathological findings	
Glomerular number per section	25 (19–32)
Global glomerulosclerosis[c], %	9.1 (2.1–19.8)
Segmental glomerulosclerosis[d], %	0.0 (0.0–2.5)
Cellular/fibrocellular crescents[d], %	0.0 (0.0–2.2)
Fibrous crescents[d], %	0.0 (0.0–0.0)
Tubulointerstitial damage, %	7.5 (5.0–20.0)
Glomerular podocin score	7.79 (7.45–7.98)
Podocyte number per glomerulus, cells/glomerulus/section	16.7 (13.7–20.7)
EGR1-positive podocytes per glomerulus, cells/glomerulus/section	0.42 (0.18–0.68)
%EGR1pod, %	2.45 (1.11–4.65)
%EGR1glo, %	29.0 (17.1–42.7)

[a]$n = 99$.
[b]$n = 96$.
[c]The percentage of global glomerulosclerosis was calculated by dividing the number of globally sclerotic glomeruli by the total number of glomeruli.
[d]The percentage of glomeruli with segmental glomerulosclerosis, cellular/fibrocellular crescents and fibrous crescents was calculated by dividing the number of glomeruli with each lesion by the number of non-globally sclerotic glomeruli.
Values are presented as the number (percentage) or median (25th–75th percentiles).
BMI, body mass index; HPF, high-power field; MPGN, membranoproliferative glomerulonephritis; PGNMID, proliferative glomerulonephritis with monoclonal IgG deposits; RAAS, renin–angiotensin–aldosterone system; RBC, red blood cell.
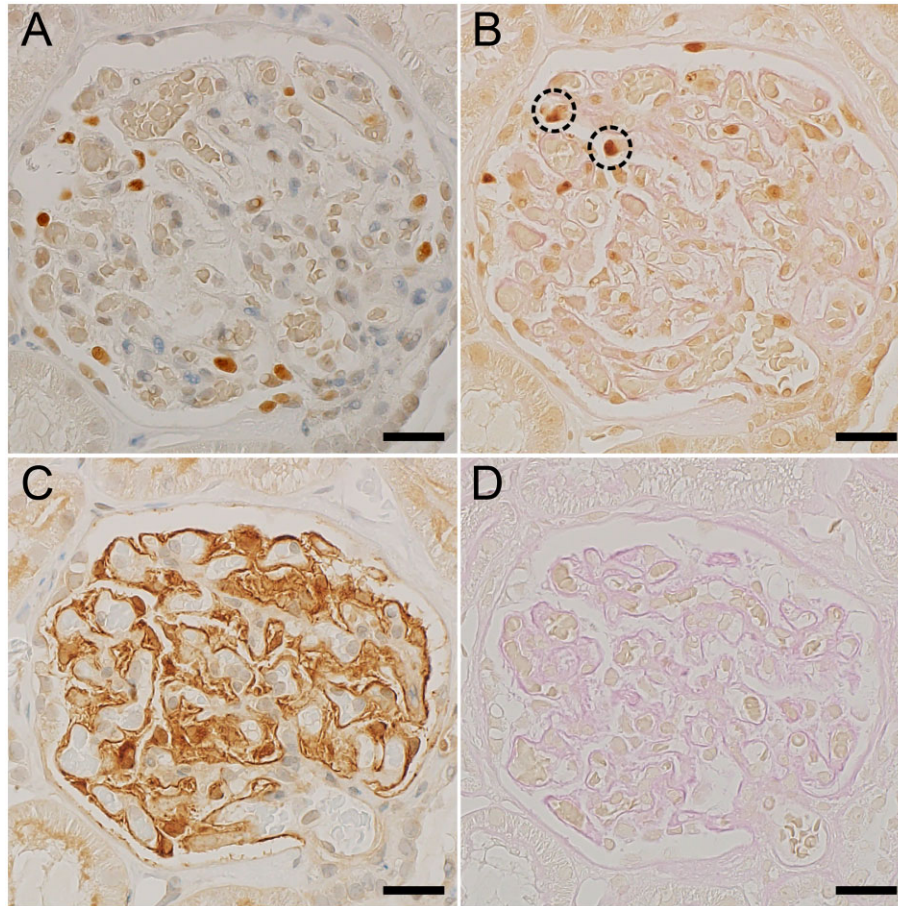
Figure 2: EGR1 expression in podocytes. Shown are representative images of WT1 (**A**), EGR1 (**B**) and podocin (**C**) staining in serial sections from the same glomerulus. The section subjected to EGR1 immunostaining were counterstained with a PAS stain. (**D**) The negative control for EGR1 staining, which was stained without the primary antibody for EGR1. The dashed circles represent EGR1-expressing podocytes. Original magnification, ×400. Scale bars = 25 μm.

Table 2: Correlation between %EGR1pod values and clinical and histopathological parameters in patients with glomerular diseases.

Variable	Rho	Unadjusted P-value	Adjusted P-value
Clinical parameters			
UPCR	0.361	<.001	.002
UPE[a]	0.342	<.001	.006
Urinary nephrin mRNA:creatinine ratio	0.514	<.001	<.001
Urinary podocin mRNA:creatinine ratio	0.487	<.001	<.001
Urinary podocalyxin:creatinine ratio	0.253	.010	.082
Urinary RBC count	0.215	.030	.211
eGFR	−0.0605	.546	1
24-h CCr[b]	−0.112	.269	1
Histopathological parameters			
Glomerular podocin score	−0.417	<.001	<.001
Podocyte number per glomerulus	−0.186	.061	.305
Cellular/fibrocellular crescents	0.479	<.001	<.001
Fibrous crescents	0.202	.042	.253
Tubulointerstitial damage	0.272	.006	.051
Global glomerulosclerosis	0.0173	.863	.863
Segmental glomerulosclerosis	0.0372	.710	1

[a]$n = 96$.
[b]$n = 99$.
Rho, Spearman's rank correlation coefficient. P-values were adjusted using the Bonferroni–Holm method.
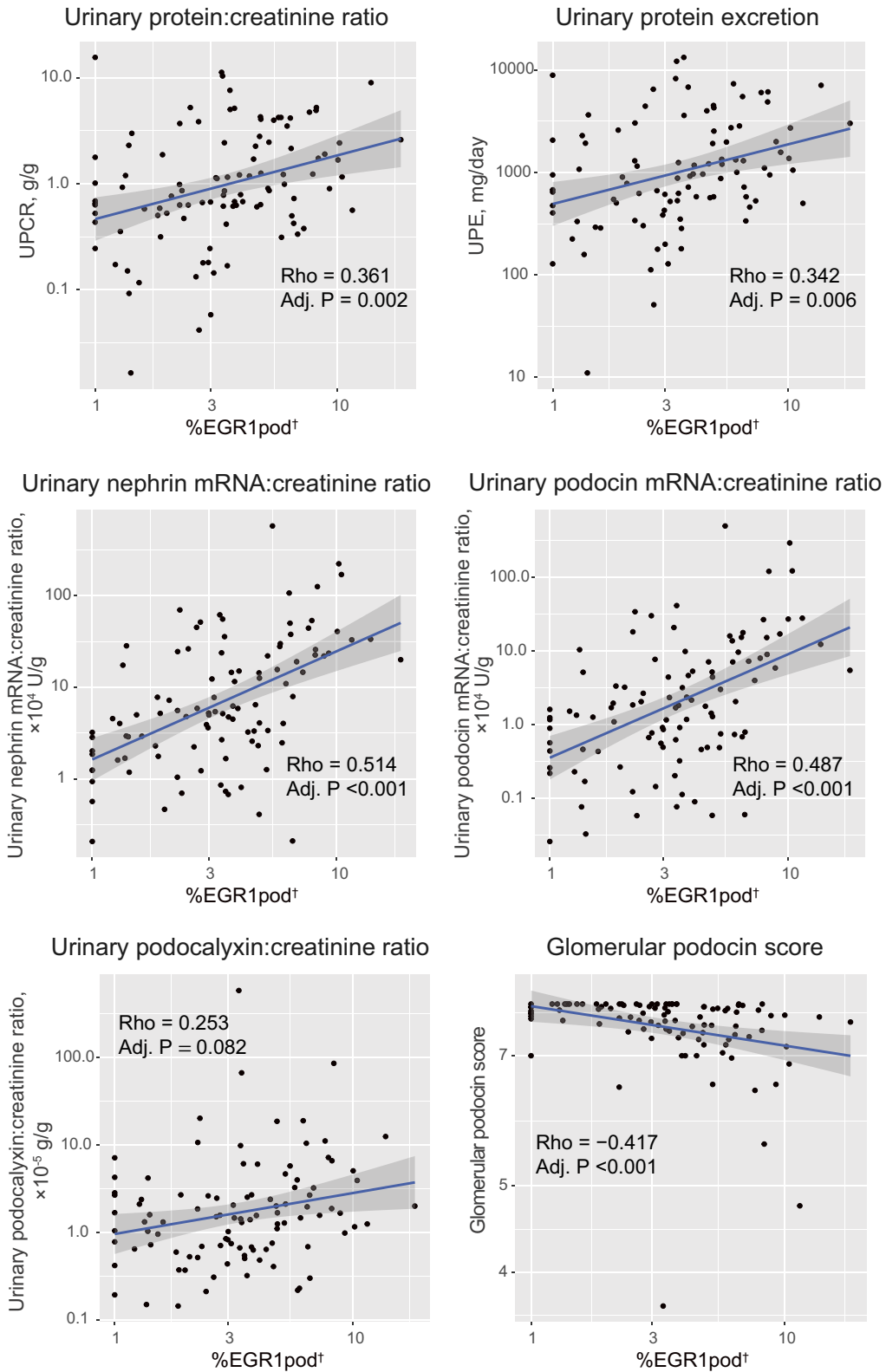RBC, red blood cell.

Figure 3: Scatterplots between %EGR1pod and podocyte biomarkers in patients with glomerular diseases. %EGR1pod correlated significantly with the UPCR, UPE, urinary nephrin mRNA:creatinine ratio, urinary podocin mRNA:creatinine and glomerular podocin score. The correlation between %EGR1pod and the urinary podocalyxin: creatinine ratio was weak, with no statistical significance. Scatterplots are depicted using linear regression and 95% confidence limits of the regression coefficient. Both axes are logarithmic. [†]Each %EGR1pod value was added to 1 for logarithmic transformation. Rho, Spearman's rank correlation coefficient; Adj. P, adjusted P-value.
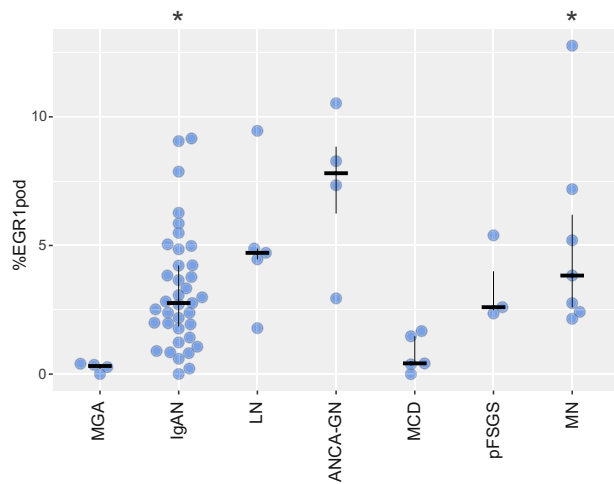
Figure 4: Comparison of podocyte EGR1 expression among glomerular diseases. Shown are the %EGR1pod values of representative glomerular diseases, including IgAN ($n = 37$), LN ($n = 5$), ANCA-GN ($n = 4$), MCD ($n = 5$), primary FSGS (pFSGS, $n = 3$) and MN ($n = 7$), as well as MGA ($n = 4$), and are depicted as circles. The %EGR1pod in IgAN and MN was significantly higher [2.76 (1.77–4.23)% and 3.83 (2.59–6.19)%, respectively; adjusted $P = .015$ and .037, respectively] than in MGA [0.31 (0.20–0.37)%]. The %EGR1pod in LN, ANCA-GN, MCD and pFSGS [4.71 (4.46–4.88)%, 7.81 (6.24–8.84)%, 0.42 (0.38–1.47)% and 2.60 (2.48–4.00)%, respectively] were not statistically significantly different from that in MGA (adjusted $P = .063$, .088, .530 and .135, respectively). Horizontal bars show the median and vertical bars indicate the 25th–75th percentiles. The P-values were adjusted using Steel's method with MGA set as a reference. *Adjusted $P < .05$.

higher %EGR1pod and higher number of podocytes was the most relevant to proteinuria remission (Fig. 6B, Supplementary data, Table S5). Although there was no statistical significance in the interaction between %EGR1pod and podocyte number, a synergistic clinical significance was observed.

Difference between podocin and EGR1 expression upon podocyte injury

Glomerular podocin expression is possibly affected by both podocyte injury and depletion; therefore, we compared glomerular podocin scores with urinary markers of podocyte injury and podocyte number. Glomerular podocin scores correlated most strongly with podocyte number per glomerulus (rho = 0.372, adjusted $P < .001$) and correlated weakly with UPCR, UPE level, and urinary nephrin and podocin mRNA levels (rho = −0.249, −0.237, −0.244 and −0.311, respectively; adjusted $P = .047$, .040, .040 and .007, respectively) (Table 4, Supplementary data, Fig. S6). The decrease in glomerular podocin expression may be more influenced by podocyte number than podocyte injury.

Identification of EGR1-positive podocytes independent of WT1 and podocin staining

In the above analyses, we identified EGR1-positive podocytes in serial sections immunostained for EGR1, WT1 and podocin. Although the number of WT1-positive podocytes was not significantly different among glomerular diseases in the present study (Supplementary data, Fig. S7), WT1 expression may be reduced beyond the level of detection in severely injured podocytes. In such cases, the %EGR1pod value may be misestimated. Therefore, we identified EGR1-positive cells located along the outer side of the GBM without relying on WT1 and podocin staining. The ratio of glomeruli containing these EGR1-positive cells (%EGR1glo) was well correlated with %EGR1pod (rho = 0.908, $P < .001$, Supplementary data, Fig. S8) and associated with proteinuria, urinary podocyte mRNA levels and cellular/fibrocellular crescents, and negatively with glomerular podocin expression (Supplementary data, Table S6 and Fig. S9). %EGR1glo could be a simple assessment tool for podocyte EGR1 expression.

Table 3: Correlation: between %EGR1pod and clinical and kidney biopsy histopathological parameters in patients with IgAN ($n = 37$).

Variable	Rho	Unadjusted P-value	Adjusted P-value
Clinical parameters			
UPCR	0.432	.008	.114
UPE[a]	0.559	<.001	.011
Urinary nephrin mRNA:creatinine ratio	0.375	.023	.184
Urinary podocin mRNA:creatinine ratio	0.4	.015	.162
Urinary podocalyxin:creatinine ratio	0.417	.011	.139
Urinary RBC count	0.156	.357	1
eGFR	−0.0242	.887	1
24-h CCr[b]	−0.00672	.969	.969
Histopathological parameters			
Glomerular podocin score	−0.362	.028	.279
Podocyte number per glomerulus	−0.177	.293	1
Endocapillary hypercellularity	0.403	.013	.161
Cellular/fibrocellular crescents	0.442	.006	.092
Fibrous crescents	0.0908	.593	1
Tubulointerstitial damage	0.361	.028	.256
Global glomerulosclerosis	−0.0327	.848	1
Segmental glomerulosclerosis	0.0988	.561	1

[a]$n = 33$.

[b]$n = 35$.

Rho: Spearman's rank correlation coefficient. P-values were adjusted using the Bonferroni–Holm method.
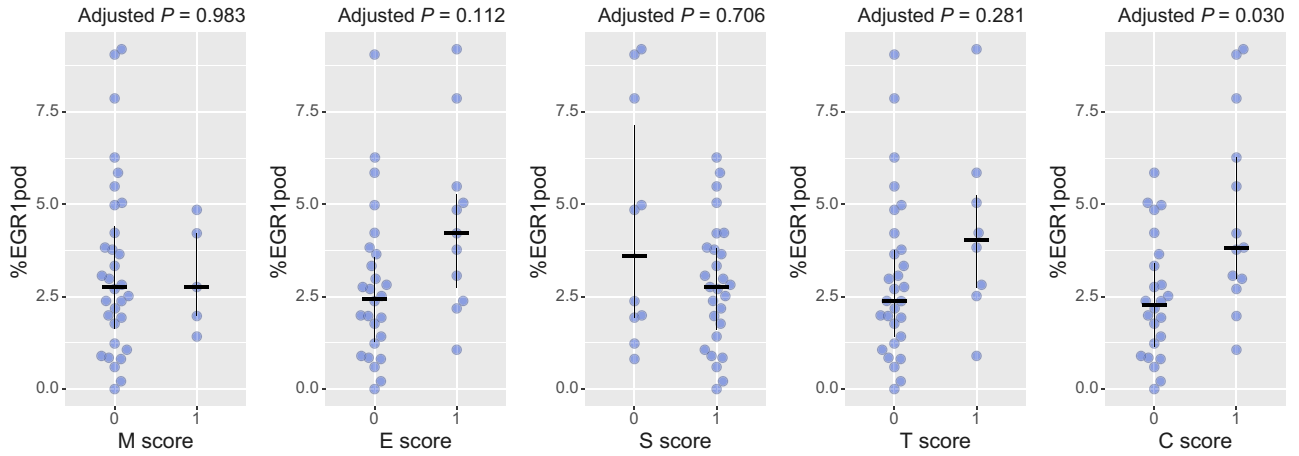RBC, red blood cell.

Figure 5: Podocyte EGR1 expression and Oxford MEST-C score in patients with IgAN. Patients with C1 lesions had significantly higher %EGR1pod than those with C0 lesions [3.83 (2.99–6.27)% vs 2.28 (1.15–3.41)%; unadjusted P = .006; adjusted P = .030]. There was no statistical difference in the M [M1 vs M0: 2.76 (1.98–4.22)% vs 2.76 (1.64–4.42)%; unadjusted P = .983; adjusted P = .983], E [E1 vs E0: 4.22 (2.72–5.26)% vs 2.45 (1.28–3.57)%; unadjusted P = .028; adjusted P = .112], S [S1 vs S0: 2.76 (1.60–3.80)% vs 3.62 (1.95–7.15)%; unadjusted P = .353; adjusted P = .706] or T lesions [T1 vs T0: 4.03 (2.75–5.24)% vs 2.39 (1.42–3.77)%; unadjusted P = .094; adjusted P = .281]. Horizontal bars show the median and vertical bars show the 25th–75th percentiles. P-values were adjusted using the Bonferroni–Holm method.
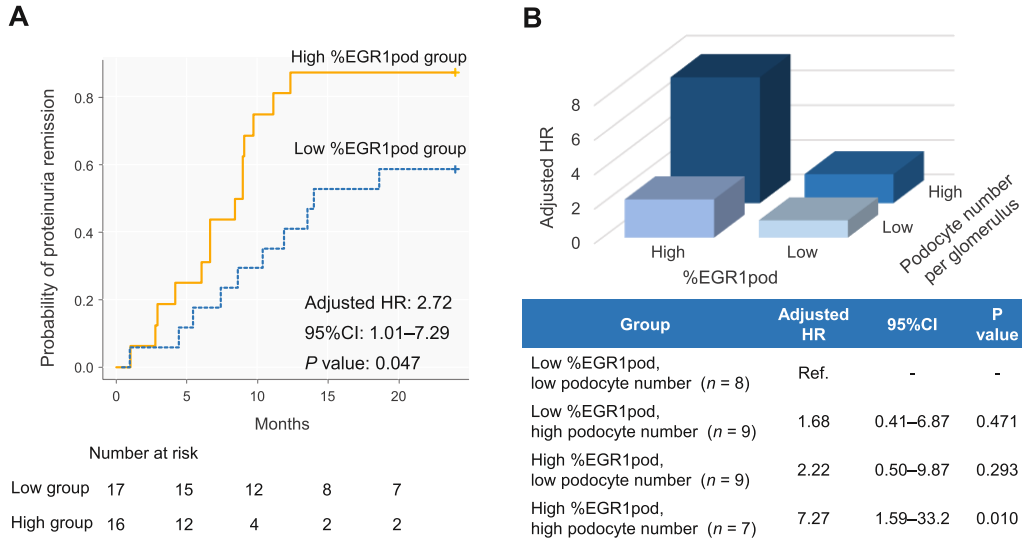

Group	Adjusted HR	95%CI	P value
Low %EGR1pod, low podocyte number (n = 8)	Ref.	-	-
Low %EGR1pod, high podocyte number (n = 9)	1.68	0.41–6.87	0.471
High %EGR1pod, low podocyte number (n = 9)	2.22	0.50–9.87	0.293
High %EGR1pod, high podocyte number (n = 7)	7.27	1.59–33.2	0.010

Figure 6: Prognostic analyses in patients with IgAN. (**A**) The probability of proteinuria remission. The patients were divided into two groups by the median of %EGR1pod (2.99%) and longitudinally observed for 2 years. Proteinuria remission was defined as at least two consecutive findings of <0.3 g/g of UPCR. The probability of protein-uria remission was estimated using a Kaplan–Meier survival curve. The HR for proteinuria remission was adjusted by age, sex, baseline UPCR, baseline eGFR and corticosteroid use. HRs and statistical significance did not change significantly with different combinations of covariates (Supplementary data, Table S4). (**B**) Subgroup analysis by podocyte number. The patients were divided into four subgroups according to the median of %EGR1pod and the median of glomerular podocyte number (15.3 cells/glomerulus/section). The HRs for proteinuria remission were adjusted by age, sex, baseline UPCR, baseline eGFR and corticosteroid use. HRs and statistical significance of the group with high %EGR1pod and high podocyte number compared to the group with low %EGR1pod and low podocyte number did not change significantly with different combinations of covariates (Supplementary data, Table S5). The P-value for the interaction between %EGR1pod and podocyte number per glomerulus was .504.

Table 4: Correlation between the glomerular podocin score and podocyte-associated markers.

Variable	Rho	Unadjusted P-value	Adjusted P-value
UPCR	−0.249	.012	.047
UPE[a]	−0.237	.020	.040
Urinary nephrin mRNA:creatinine ratio	−0.244	.013	.040
Urinary podocin mRNA:creatinine ratio	−0.311	.001	.007
Urinary podocalyxin:creatinine ratio	−0.122	.222	.222
Podocyte number per glomerulus	0.372	<.001	<.001

[a]n = 96.
Rho, Spearman's rank correlation coefficient. P-values were adjusted using the Bonferroni–Holm method.

DISCUSSION

Strategies to protect podocytes from injury before depletion are desirable to prevent the onset and progression of glomerular diseases, as podocytes cannot replicate. In this regard, visualizing injured podocytes that have not been shed from the glomeruli is crucial; however, to date, no established markers that are enhanced in injured podocytes in human tissues exist. Although Wnt/beta-catenin and its transcripts and filamin-B may be candidates for such markers [26–28], none has been validated in human glomerular diseases. In this study, we discovered that podocyte EGR1 expression correlated significantly with proteinuria and urinary podocyte mRNA levels and inversely with glomerular podocin expression. Moreover, EGR1 was also occasionally expressed in binucleated podocytes, which are known to be associated with podocyte injury [29]. These results suggest that EGR1 is a pathological marker of podocyte injury in human glomerular diseases.

Immunostaining analysis of podocyte-associated proteins is often performed to evaluate podocyte injury; however, podocyte injury cannot be distinguished from depletion. In this study, glomerular podocin staining correlated most strongly with podocyte number per glomerulus and correlated weakly with proteinuria, and urinary nephrin and podocin mRNA levels, while podocyte EGR1 expression did not correlate with podocyte number. Furthermore, markers that are *de novo* expressed by injury are easier to identify than those that are downregulated by injury. These findings imply that EGR1 may be superior to podocin for detecting ongoing podocyte injury.

Podocyte EGR1 expression was significantly associated with acute extracapillary lesions and was more frequently observed in the patients with ANCA-GN and LN, which are prone to crescentic glomerulonephritis. Podocyte EGR1 expression was also higher in IgAN patients with Oxford C1 lesions than in those without C lesions. Since podocyte injury has been reported to be associated with active lesions and acute extracapillary proliferation in patients with glomerulonephritis, including IgAN, ANCA-GN and LN, podocyte EGR1 expression could reflect secondary podocyte injury due to active glomerular inflammation [9, 11, 13–15, 30]. Podocyte injury may have been observed more frequently in patients with ANCA-GN and LN than those with other glomerular diseases because of the potential for rapid progression to glomerulosclerosis. In fact, urinary podocyte mRNAs were reported to be higher in LN and crescentic glomerulonephritis than in other glomerular diseases [15]. The lack of a relationship between %EGR1pod and kidney function or glomerulosclerosis among all patients might be due to the heterogeneity of glomerular diseases.

Notably, the longitudinal analysis of IgAN patients showed that a higher %EGR1pod was relevant to proteinuria remission. This association could be more pronounced in patients with higher podocyte number, despite statistical evidence in this small group. Given that most of these patients were intensively treated with corticosteroids, EGR1-expressing injured podocytes in active IgAN could be rescued from depletion by anti-inflammatory treatment. This may be consistent with the fact that urinary podocyte markers decrease after treatment in IgAN patients [13]. Intensive treatment in patients showing active glomerulonephritis with EGR1-positive podocytes could improve kidney prognoses. Further studies on various types of glomerulonephritis are warranted.

Among primary podocytopathies, EGR1-expressing podocytes were less frequently observed in MCD than in MN and primary FSGS. Although MCD often relapses, it has a better kidney prognosis than in MN or FSGS [31, 32]. Unlike secondary podocyte injury in glomerulonephritis, podocyte EGR1 expression may be associated with poor prognosis in primary podocytopathies where injury is relatively confined to podocytes. Also, differentiating MCD from primary FSGS is often difficult at the time of diagnosis, but EGR1 expression could assist in this regard. Further studies are required to clarify these.

The role of EGR1 in podocyte injury has not been fully elucidated. Recent studies have demonstrated that EGR1 induces podocyte injury via the direct regulation of thioredoxin-interacting protein (TXNIP), which is necessary for Nod-like receptor protein 3 (NLRP3) inflammasome activation and promotes oxidative stress [33, 34]. Both NLRP3 inflammasome and oxidative stress have been observed in podocytes of human glomerular diseases and animal models [35–40] and represent possible mechanisms of podocyte injury related to EGR1. Another possible mechanism is the antagonistic effect of EGR1 against WT1, leading to the dedifferentiation of podocytes [20]. EGR1 and WT1 both belong to the EGR family and share highly homologous DNA binding domains, and competition between EGR1 and WT1 has been reported in other cells [41–44]. Podocyte EGR1 expression has been reported to be regulated by epigenetic modifications, metabolites and cytokines [21, 33, 34, 45, 46]; these factors might affect podocyte EGR1 expression in podocytopathies.

A limitation of this study is that EGR1 expression is not specific to podocytes. EGR1 has been previously reported to be expressed in mesangial cells, renal tubules and the interstitium [47–50]. Therefore, to identify EGR1-expressing podocytes, we performed PAS staining to detect GBM and immunostaining for WT1 and podocin in serial sections. Another limitation is that %EGR1pod was calculated using serial paraffin sections, which may have led to measurement errors between sections. To minimize these differences, we calculated %EGR1pod using total EGR1 and WT1 counts in all glomeruli of the sections instead of the average EGR1:WT1 ratio in each glomerulus. Moreover, %EGR1glo, which is calculated with one section of EGR1 staining, showed a strong correlation and similar results with %EGR1pod. However, underestimation of EGR1-positive podocytes in glomeruli with segmental crescents is possible.

In conclusion, EGR1 may serve as an effective marker for identifying active early podocyte injury in human glomerular diseases.

SUPPLEMENTARY DATA

Supplementary data are available at *ckj* online.

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AUTHORS' CONTRIBUTIONS

M.O., T.M. and T.Y. were involved in the conception and design of the experiments. M.O. and K.K. performed experiments. M.O., K.K., I.Y. and N.T. analyzed data and interpreted experimental results. M.O. prepared tables and figures and drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author.

CONFLICT OF INTEREST STATEMENT

All authors report no disclosures.

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