

Original Article

## Glomerular glucocorticoid receptor expression is reduced in late responders to steroids in adult-onset minimal change disease

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### Abstract

**Background.** Compared to children, adult patients with minimal change disease (MCD) tend to have a slower response to steroids, but little is known about the factors influencing the steroid responsiveness in these patients. In this study, we investigated the difference in the expression of the glomerular glucocorticoid receptor (GCR) according to steroid responsiveness in 28 adult-onset MCD patients.

**Methods.** Based on the response to steroid treatment, the patients were divided into early responders (ER,  $n = 20$ ) and late responders (LR,  $n = 8$ ) according to the response to steroids on the basis of 4 weeks of treatment. The clinical and laboratory findings, and the glomerular mRNA and protein expression of GCR and nephrin, assessed by real-time polymerase chain reaction and immunohistochemistry, respectively, were compared between the ER and LR groups. Ten microscopic haematuric patients in whom renal biopsy was performed and revealed no histological abnormalities were included for control (C).

**Results.** The mRNA expression of GCR was significantly lower in the LR than that in the ER group ( $P < 0.01$ ), whereas it was comparable between the C and ER groups. GCR protein expression was also decreased in the LR compared with the C and ER groups. In contrast, there was no significant difference in nephrin mRNA expression among the three groups. On the other hand, the GCR mRNA expression correlated inversely with the time to complete remission ( $r = -0.49$ ,  $P < 0.05$ ), but not with the amount of proteinuria at presentation.

**Conclusion.** In conclusion, the levels of glomerular GCR expression may be a useful predictor of steroid responsiveness in adult-onset MCD patients.

**Keywords:** glucocorticoid receptor; minimal change disease; real-time PCR; steroid responsiveness

### Introduction

Minimal change disease (MCD) is the most common pathological finding of nephrotic syndrome in children, accounting for more than 75% of cases, whereas only 20–30% of adult patients with nephrotic syndrome have MCD [1]. In general, three-fourths of the children with MCD achieve remission within 2 weeks, and roughly 95% within 8 weeks from starting steroid treatment [2,3]. On the other hand, adult-onset MCD patients have a tendency to respond to steroids slowly, requiring longer duration of high-dose glucocorticoid treatment than children with MCD [4,5].

Glucocorticoid is the drug of choice for patients with MCD, but in whom its action mechanism is still unclear. Glucocorticoid exerts its action by binding to the intracellular glucocorticoid receptor (GCR) [6]. The number of GCR varies in different organs and the expression of GCR changes in different pathological states [7–9]. Previous study has demonstrated that the efficacy of glucocorticoid treatment on tumour cells correlated with the density of GCR in lymphoid leukaemia and lymphoma [10]. In children with nephrotic syndrome, there have also been studies on the relationship between the density of GCR in mononuclear leukocytes and steroid responsiveness [11,12], but the results were not consistent.

Nephrin, a product of the NPHS1 gene that is mutated in patients with congenital nephrotic syndrome of the Finnish type [13], was the first protein demonstrated to comprise the slit diaphragm.

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A reduction in nephrin expression was observed in experimental glomerular diseases [14,15] and in adult patients with primary acquired nephrotic syndrome [16–18], suggesting that a decrease in nephrin expression may reflect podocyte injury.

Since side-effects develop in a high proportion of patients with prolonged glucocorticoid treatment, early combined therapy with a second-line drug will be beneficial in patients whose response to glucocorticoids is expected to be unfavourable. It has been reported that age [19] and renal function at onset, selectivity of proteinuria and relative interstitial volume are associated with the steroid responsiveness in adult MCD patients [4]. However, little is known about the relationships between the glomerular expression of certain genes and the response to glucocorticoid treatment in adult patients with MCD.

To determine whether steroid responsiveness is dependent on the amount of glomerular GCR expression and the extent of podocyte injury, we compared the expression of GCR and nephrin in microdissected glomeruli obtained from adult MCD patients according to the response to glucocorticoids. In addition, the correlations between the time interval from the start of glucocorticoid therapy to complete remission (CR) and the glomerular expression of GCR and nephrin were analysed.

## Patients and methods

Twenty-eight adult patients with MCD confirmed by renal biopsy for the first time in Yonsei Medical Center, Seoul, Korea, between January 2001 and December 2005 were included. MCD was diagnosed by a single pathologist if there were no or only minimal histological changes on light microscopy, no immunofluorescence within the glomeruli, and diffuse effacement of foot processes of podocytes without electron-dense deposits. Any patient who had underlying secondary causes of MCD, concomitant glomerular or interstitial pathology on renal biopsy, aged less than 15, or who had been followed up for <6 months was excluded. In addition, patients who were on glucocorticoid treatment at the time of renal biopsy were also excluded. Baseline demographic data (age, sex, and comorbidities, etc.) and laboratory findings (urinalysis, haemoglobin, blood urea nitrogen, serum creatinine, serum albumin, serum cholesterol, 24 h urinary protein excretion, urinary protein to creatinine ratio, selective proteinuria index, etc.) were collected by retrospective review of the medical records of patients.

All patients were initially treated with oral prednisolone in a single dose of 1.0 mg/kg/day and gradual tapering of the dosage was done if CR was achieved. The time interval from the start of glucocorticoid treatment to CR was calculated. CR was defined as negative or trace on urine dipstick tests and urinary protein to creatinine ratio <0.3 mg/mg without oedema. Based on the response to glucocorticoid treatment, the patients were divided into two groups: early responders (ER) in whom CR was achieved within 4 weeks of steroid treatment; late responders (LR) in whom CR was achieved after 4 weeks of steroid treatment.

A comparative analysis of the clinical and biochemical findings was performed between the ER and LR groups. In addition, the expression of GCR, nephrin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in microdissected glomeruli obtained at the time of biopsy, assessed by real-time polymerase chain reaction (PCR) and immunohistochemistry, were compared between the two groups. Ten microscopic haematuric patients in whom renal biopsy was performed and revealed no histological abnormalities were included for controls (C), and glomerular GCR mRNA and protein expression in the C group were also compared with those in the ER and LR groups. This study was approved by the Institutional Review Board for human research at Yonsei University College of Medicine and informed consent was obtained from all patients.

### Microdissection and reverse transcription

Microdissection of glomeruli was performed as previously described [20]. Glomeruli were microdissected at 4°C in vanadyl ribonucleoside complex, rinsed, aliquoted four per tube in RNase inhibitor, solubilized in triton buffer, and lysed by freezing and thawing.

First-strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Glomeruli were directly reverse transcribed using 10 µM random hexanucleotide primer, 1 mM dNTP, 8 mM MgCl<sub>2</sub>, 30 mM KCl, 50 mM Tris-HCl, pH 8.5, 0.2 mM dithiothreitol, 25 U RNase inhibitor and 40 U AMV reverse transcriptase. The mixture was incubated at 30°C for 10 min and 42°C for 1 h followed by inactivation of the enzyme at 99°C for 5 min.

### Real-time PCR

Real-time PCR was performed to determine the mRNA expression of GCR, nephrin and GAPDH. The primers used in this study were as follows: GCR sense 5'-GCAGCGAAGACTTTTGGTTG-3', antisense primer 5'-GTGAAACTGCTTTGGACAGA-3'; nephrin sense 5'-GGTGAATTCCACCTGCACATC-3', antisense 5'-AGCTTGGGAGGAACCAGGAT-3'; and GAPDH sense 5'-GATTCCACCCATGGCAAAT-3', antisense 5'-AGATGGGTGATGGGATTCCATT-3'. Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), PCR was performed with a total volume of 20 µl in each well, containing 10 µl of SYBR Green® PCR Master Mix (Applied Biosystems), 5 µl of cDNA corresponding to one-tenth of a glomerulus and 5 pmol sense and antisense primers. Primer concentrations were determined by preliminary experiments that analysed the optimal concentrations of each primer. Each sample was run in triplicate in separate tubes. The PCRs were performed with 35 cycles of denaturation at 94.5°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 1 min. Initial heating at 95°C for 9 min and final extension at 72°C for 7 min were performed for all PCRs.

After PCR, the temperature was increased from 60°C to 95°C at a rate of 2°C/min to construct a melting curve. A control without cDNA was run in parallel with each assay. The cDNA content of each specimen was determined using a comparative C<sub>T</sub> method. The results are given as relative expression of nephrin and GCR normalized to GAPDH and

are expressed as  $-\Delta\Delta C_T$ . In pilot experiments, PCR products run on agarose gels revealed a single band.

### Immunohistochemistry

Biopsy specimens fixed in 10% neutral buffered formalin were used for immunohistochemical staining. Four micron sections on the slides were deparaffinized, hydrated in ethyl alcohol and washed in tap water. Antigen retrieval was carried out in 10mM sodium citrate buffer for 20 min using a vegetable steamer. For GCR staining, a rabbit polyclonal anti-GCR antibody (EMD Biosciences, Inc., Darmstadt, Germany) was diluted in 1:100 with 2% casein in BSA and was applied for overnight incubation at room temperature. After washing, a secondary goat anti-rabbit antibody (Inc., Santa Cruz, Ca, USA) was added for 20 min, the slides were washed and incubated with a tertiary rabbit-PAP complex (Santa Cruz Biotechnology, Inc.) for 20 min. Diaminobenzidine was added for 2 min and the slides were counterstained with haematoxylin. A semi-quantitative score for measuring intensity of staining within glomeruli was determined by examining all glomeruli (mean,  $9.3 \pm 1.2$  glomeruli; range, 5–18 glomeruli) in each section and by digital image analysis (MetaMorph version 4.6r5, Universal Imaging Corp., Downingtown, PA, USA) as previously described [21].

### Statistical analysis

All values are expressed as the mean  $\pm$  SEM. Statistical analysis was performed using the statistical package SPSS for Windows Ver. 13.0 (SPSS, Inc., Chicago, IL, USA). Results were analysed using the chi-square test or Kruskal–Wallis non-parametric test for multiple comparisons. Significant differences by the Kruskal–Wallis test were further confirmed by the Mann–Whitney U test. Correlation between the mRNA expression of GCR and the time interval from the start of steroid treatment to CR was determined by Spearman correlation analysis. Statistical significance was determined, when *P*-values were less than 0.05.

## Results

### Demographic characteristics and laboratory findings

Among 28 patients with MCD, 17 were men and 11 were women, with the mean age at the time of renal biopsy  $32.5 \pm 2.4$  years. There were two patients with hypertension and three with renal failure (serum creatinine more than 1.5 mg/dl) at presentation. Renal failure in all the three patients was considered a consequence of effective circulating volume depletion, based on the results of urinary indices and renal biopsy and in terms of complete recovery by conservative treatment. The ER group consisted of 20 patients (71.4%) and the LR group of 8 patients (28.6%). The time interval from the start of steroid treatment to CR was significantly shorter in the ER group than that in the LR group ( $16.5 \pm 0.9$  vs  $52.8 \pm 4.9$  days,  $P < 0.005$ ). On the other hand, there were no differences in age, sex ratio, patients with

**Table 1.** Comparison between ER and LR

	Control ( <i>n</i> = 10)	ER <sup>a</sup> ( <i>n</i> = 20)	LR <sup>b</sup> ( <i>n</i> = 8)
Time to CR (days)	–	$16.5 \pm 0.9$	$52.0 \pm 4.9^{\#}$
Age at onset	$33.4 \pm 3.8$	$32.3 \pm 2.7$	$32.9 \pm 5.3$
Male/Female	6/4	13/7	4/4
Hypertension	1	1 (5.0%)	1 (12.5%)
Microscopic haematuria	10	1 (5.0%)*	3 (37.5%)*
Renal failure at presentation	0	2 (10.0%)	1 (12.5%)
Haemoglobin (g/dl)	$14.8 \pm 0.2$	$14.5 \pm 0.3$	$13.4 \pm 0.8$
Albumin (g/dl)	$4.3 \pm 0.5$	$1.8 \pm 0.4^*$	$2.1 \pm 0.6^*$
BUN (g/dl)	$13.7 \pm 2.7$	$21.0 \pm 2.9$	$19.3 \pm 5.4$
Creatinine (mg/dl)	$1.0 \pm 0.1$	$1.1 \pm 0.1$	$1.2 \pm 0.2$
Total cholesterol (mg/dl)	$165.4 \pm 21.7$	$387.7 \pm 22.3^*$	$348.3 \pm 42.4^*$
24 h urinary protein excretion (g/24 h)	–	$11.5 \pm 3.1$	$9.6 \pm 3.4$
Selective protein index (SPI)	–	$0.16 \pm 0.04$	$0.19 \pm 0.04$
Serum IgG (mg/dl)	$897.1 \pm 107.2$	$415.1 \pm 48.3$	$665.3 \pm 280.2$
Serum IgA (mg/dl)	$211.1 \pm 21.4$	$283.0 \pm 30.8$	$194.7 \pm 27.7$
Serum IgM (mg/dl)	$128.1 \pm 16.4$	$145.5 \pm 17.1$	$157.3 \pm 18.2$
C3 (mg/dl)	$124.8 \pm 5.4$	$122.6 \pm 5.9$	$115.9 \pm 6.2$
C4 (mg/dl)	$38.6 \pm 7.1$	$48.0 \pm 14.5$	$32.4 \pm 5.6$

Values are expressed as mean  $\pm$  SEM. <sup>a</sup>ER, early responders, CR achieved within 4 weeks of steroid therapy, <sup>b</sup>LR, late responders, CR achieved after 4 weeks of steroid therapy.

\* $P < 0.01$  vs control, <sup>#</sup> $P < 0.05$  vs ER.

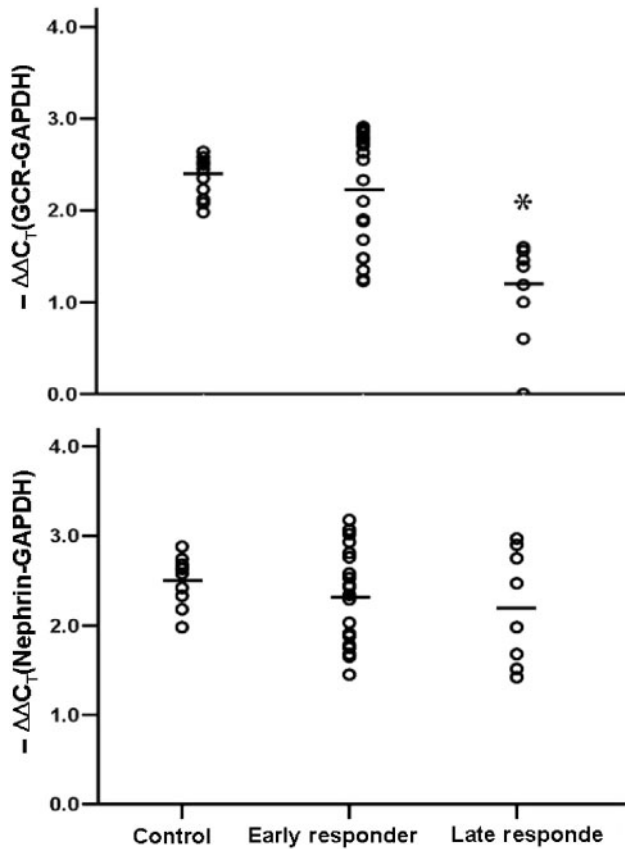
hypertension or renal failure and laboratory findings such as haemoglobin, blood urea nitrogen, serum creatinine, serum albumin, serum cholesterol, 24 h urinary protein excretion, selective proteinuria index between the two groups (Table 1).

### mRNA expression according to steroid responsiveness

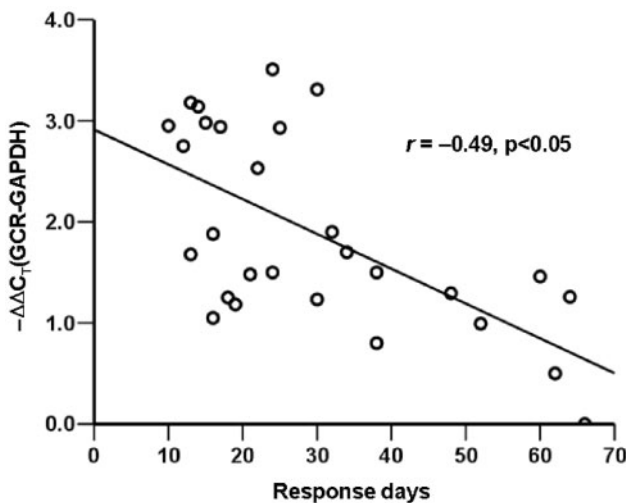
Real-time PCR revealed that the GCR mRNA expression normalized to GAPDH, which was expressed as  $-\Delta\Delta C_T$ , was significantly lower in the LR group ( $1.25 \pm 0.55$ ) compared to the ER group ( $2.17 \pm 0.61$ ) ( $P < 0.01$ ), whereas it was comparable between the C ( $2.35 \pm 0.23$ ) and ER groups (Figure 1A). In contrast, there was no significant difference in nephrin mRNA expression among the three groups (Figure 1B). On the other hand, the GCR mRNA expression correlated inversely with the time to CR ( $r = -0.49$ ,  $P < 0.05$ ) (Figure 2), but not with the amount of proteinuria at presentation (Figure 3).

### Immunohistochemical staining for GCR

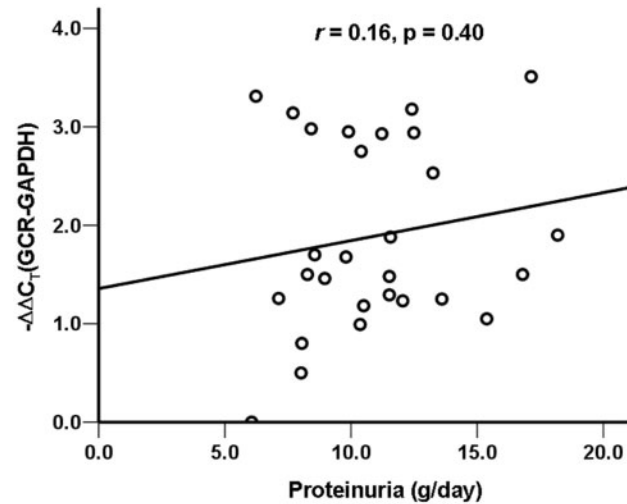
GCR protein expression in glomeruli, assessed by immunohistochemical staining, showed a similar pattern to the observed changes in the GCR mRNA expression. The staining for GCR within glomeruli was strong in cells with the location and appearance of podocytes (Figure 4A). The mean semi-quantitative staining scores for glomerular GCR were significantly



**Fig. 1.** GCR/GAPDH mRNA (A) and nephrin/GAPDH mRNA (B) in the C, and ER and LR groups. Real-time PCR revealed that GCR mRNA expression normalized to GAPDH, which was expressed as  $-\Delta\Delta C_T$ , was significantly lower in the LR group compared with the ER group, whereas nephrin mRNA expression normalized to GAPDH was comparable between the two groups. The GCR/GAPDH mRNA and nephrin/GAPDH mRNA ratios in the control group were similar to those in the ER group. \* $P < 0.01$  vs control and ER groups.



**Fig. 2.** Correlation between the GCR mRNA expression and time interval from the start of steroid therapy to complete remission. There was a significant inverse correlation between the mRNA expression of GCR and the time interval from the start of steroid treatment to complete remission.



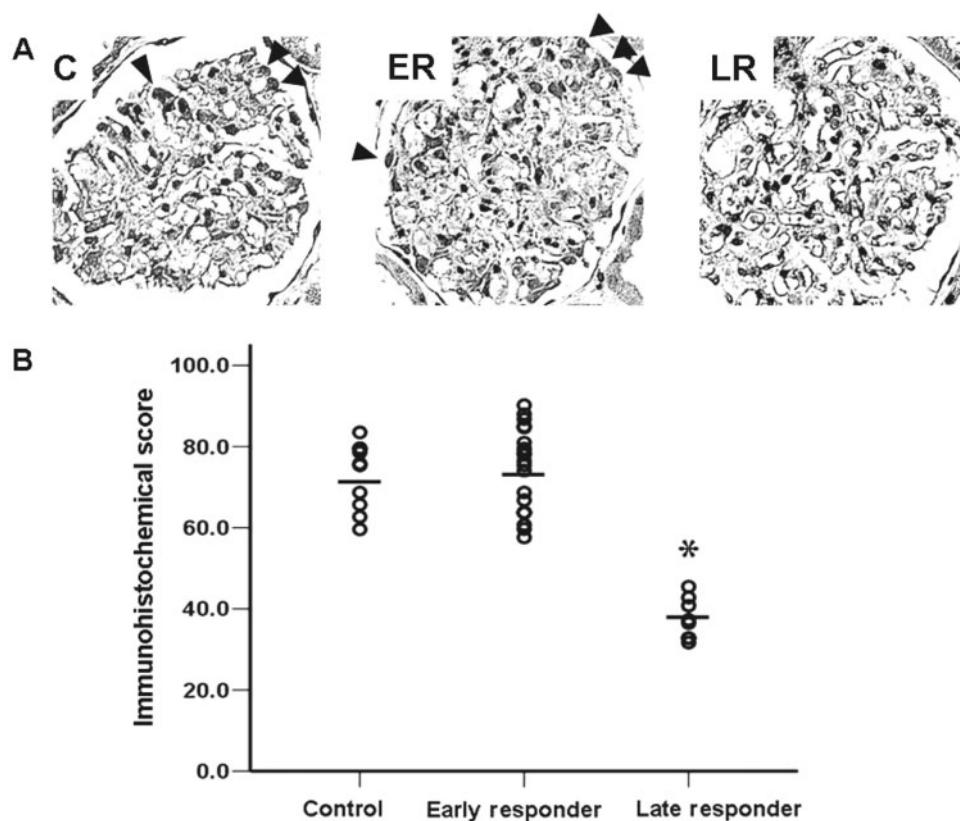
**Fig. 3.** Correlation between the GCR mRNA expression and the amount of proteinuria at presentation. There was no significant correlation between the mRNA expression of GCR and the amount of proteinuria.

lower in the LR group ( $37.5 \pm 5.1$ ) compared to the C ( $72.9 \pm 8.1$ ) and ER groups ( $74.1 \pm 10.9$ ) ( $P < 0.05$ ) (Figure 4B).

## Discussion

In this study, we show for the first time that glomerular GCR expression is decreased in adult MCD patients whose response to glucocorticoids is delayed. In addition, this study shows that the time interval from the start of steroid treatment to CR was inversely correlated with the mRNA expression of GCR in glomeruli.

MCD is the predominant form of nephrotic syndrome in children, most of whom promptly respond to glucocorticoid [1–3]. Even though the overall response rates to steroids in adult MCD patients are similar to those in children, the time to remission is known to be longer in adult-onset MCD compared with the childhood group [4–5]. It is not clear why the response to steroids is slower in adult patients with MCD. To clarify factors influencing the pattern of response to steroid therapy, Nakayama *et al.* [4] compared the characteristics and histological findings between early and late responders. They demonstrated that the incidence of microscopic haematuria and levels of both blood urea nitrogen and serum creatinine were significantly higher in late compared to early responders. In addition, late responders had poorer selectivity of proteinuria and greater relative interstitial volume than early responders. In this study, there were no differences in the proportion of patients with microscopic haematuria, blood urea nitrogen, serum creatinine or selective proteinuria index between the ER and LR groups. The reason for the divergence of results may be due to the criteria for defining early



**Fig. 4.** (A) A representative immunohistochemical staining for GCR protein in the C, and ER and LR groups. The staining for GCR within glomeruli was strong in cells with the location and appearance of podocytes (arrow heads). (400 $\times$ ) (B) The mean semi-quantitative staining scores for glomerular GCR were significantly lower in the LR group compared with the C and ER groups. \* $P < 0.05$  vs C and ER groups.

and late responders (the time to remission: 4 vs 8 weeks). Compared to the study by Nakayama *et al.* [4], the response rates to steroids at the corresponding time course were higher and only three patients achieved CR after 8 weeks of steroid treatment in this study. Therefore, we could not classify the subjects according to the same method as Nakayama *et al.* [4]. Huang *et al.* [22] also divided the patients into early and late responders according to the response to glucocorticoids on the basis of 4 weeks as in this study, and compared the frequency of relapse, but did not investigate the differences in clinical or laboratory findings between the two groups.

Even though abnormal immune function, especially disturbances in T-cell function, has been supposed to be implicated in the pathogenesis of MCD [23], the detail action mechanisms of glucocorticoid in this disease have not been clearly explored. Glucocorticoids act by binding to intracellular GCR, which results in the formation of glucocorticoid–GCR complex, and then this complex is translocated to the nucleus and binds to the DNA and acts as a transcription factor [6]. The number of GCR varies in different pathological states and individual differences also exist in the expression of GCR. Previous studies have demonstrated that the concentrations of GCR in peripheral mononuclear leukocytes were closely related to the steroid responsiveness in various autoimmune diseases such as rheumatoid arthritis [7], lupus nephritis [8] and

asthma [9]. Based on the possibility of the involvement of cellular immune disturbance in the pathogenesis of MCD, Bagdasarova *et al.* [11] examined the association of GCR number in monocytes and steroid responsiveness in patients with nephrotic syndrome and reported a higher expression of GCR in steroid-sensitive than in steroid-resistant patients. In contrast, Haack *et al.* [12] did not find any difference in density and binding affinity of GCR in mononuclear leukocytes between steroid-sensitive and steroid-resistant nephrotic syndrome children and raised a probability that steroid sensitivity may depend on other factors such as the amount of GCR in renal tissue.

In fact, there have been some reports that changes in the expression of GCR in target tissue may play an important role in steroid resistance in various diseases. Inhibition of GCR expression by oestradiol induced steroid resistance in human breast cancer cells [24] and transformed mouse lung cells stably transfected with GCR gene acquired glucocorticoid responsiveness [25]. Moreover, the efficacy of glucocorticoid treatment on tumour cells correlated with the density of GCR in lymphoid leukaemia and lymphoma [10]. In kidney, GCR is known to be widely distributed with intense expression in distal convoluted tubules and collecting ducts, faint expression in proximal tubules, and moderate expression in glomerular cells, including mesangial cells, endothelial cells and podocytes [26] and potentially mediates the pharmacological effects of

synthetic glucocorticoid. Recent study also demonstrated that dexamethasone not only up-regulated nephrin and tubulin- $\alpha$  expression but also suppressed cytokine production in human podocytes, suggesting a potent effect of glucocorticoids directly on podocytes [27]. However, there has been no study on the relationship between the expression of glomerular GCR and the sensitivity to steroids in patients with nephrotic syndrome so far. In this study, we demonstrated for the first time that glomerular GCR expression was lower in late compared with early responders and was negatively correlated with the time interval to remission.

Podocyte injury is a key component of the process in various glomerular diseases characterized by proteinuria. The underlying pathological change responsible for glomerular proteinuria is the loss of size-selective and/or charge-selective properties of the glomerular filtration barrier [28], and nephrin is known to serve as a basic scaffold for the slit diaphragm and play a central role in the glomerular filtration barrier [29,30]. Recent studies have demonstrated that nephrin expression was reduced in experimental and human proteinuric diseases [16–18], suggesting that a decrease in nephrin expression may reflect podocyte injury. For this reason, we speculated that severer injury to podocytes expressed as more decreased expression of nephrin may require longer treatment duration to induce remission. The mRNA expression of nephrin, however, was not different between the ER and LR groups. Moreover, its expression was comparable between C and patients with MCD. Further study will be needed to clarify whether decreased nephrin expression is a real sensitive marker of podocyte injury and whether podocyte injury is involved in the pathogenesis of MCD.

In conclusion, glomerular GCR expression was significantly lower in late than in early responders and there was a significant inverse correlation between the expression of GCR and the time interval from the start of steroid treatment to CR. These findings suggest that the levels of GCR expression may be useful predictors of steroid responsiveness in adult-onset MCD patients.

*Conflict of interest statement.* None declared.

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