

Soluble Interleukine-2 Recptor and *MDR1* Gene Expression Levels as Inflammatory Biomarkers for Prediction of Steroid Response in Children With Nephrotic Syndrome

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Introduction. Upregulation of interleukin-2 may be involved, not only in the pathophysiology of nephrotic syndrome, but also in steroid resistance treatment, by increasing expression of multidrug resistant gene-1 (*MDR1*) gene on lymphocytes and its product P-glycoprotein effluxing corticosteroid. Our aim was to assess the relation of serum soluble interleukin-2 receptor (sIL2R) levels and *MDR1* gene expression on lymphocytes with nephrotic syndrome and its corticosteroids therapy.

Materials and Methods. We examined 40 children with nephrotic syndrome (15 cases of recent onset and 25 known cases with relapse) and 20 healthy children as a control group. We examined every patient twice at the time of disease activity and within 1 week of remission.

Results. A significant increase was found in sIL2R level and *MDR1* gene in the patients in comparison with the control group whether in activity or remission, and they were significantly higher in activity than in remission. Levels of sIL2R and *MDR1* gene expression in different subgroups were higher in known cases with relapse than in new onsets, both in activity and remission, and relatively higher in steroid-resistant than in steroid-sensitive ones.

Conclusions. We propose sIL2R and *MDR1* gene expression levels as early predictors of steroid resistance in nephrotic syndrome for early control of disease by immediate introduction of cytotoxic drugs. This is the first report providing new insight into the use of sIL2R as a predictor of steroid resistance. Thus, wide-scale studies are needed to determine a cutoff level of sIL2R above which cytotoxic drugs are introduced.

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INTRODUCTION

Idiopathic nephrotic syndrome (NS) is currently considered an immune-mediated disease related to T-cell disorders.¹ After initiation of T-cell activation by the role of the T-cell receptor, intermediate proteins are phosphorylated and dephosphorylated to activate transcription factors,

which are translocated to the nucleus to activate the transcription of set of genes such as interleukin-2 (*IL2*). Interleukin-2 (*IL-2*) can be recycled by binding to the *IL2* receptor, then signaling through the Jak-3 tyrosine kinase to promote a long-term proliferation of activated T cells,² thus allowing the release of a soluble component of the *IL-2* receptor

(sIL2R) into the circulation.³ Increased expression of *IL2* mRNA with subsequent increase in sIL2R in the acute phase of idiopathic NS has suggested that they, at least in part, might be involved in the pathophysiology of childhood idiopathic NS.³

It has recently been postulated that *IL2* induces increments of multidrug resistance protein 1 (*MDR1*) expression through a translocation of *MDR1*-specific transcriptional factor, Y-box protein-1, from cytoplasm to nuclei in lymphocytes.⁴ Some studies have provided evidence that upregulation of IL-2 and its compound (sIL2R) may be involved not only in the pathophysiology of NS, but also in steroid resistance to treatment by increasing expression of *MDR1* gene and its product, P-glycoprotein. P-glycoprotein-170, a product of the *MDR1* gene on chromosome 7p21, is a member of the ATP-binding cassette transporter superfamily of genes, and it functions as an energy-dependent transmembrane efflux pump.⁵

Overexpression of P-glycoprotein results in reduction of intracellular concentrations of xenobiotics, drugs, and poisons, such as vinca alkaloids, anthracyclines, verapamil, and corticosteroids, which are mainly substances whose molecular weights range from 300 to 2000 Da.⁶ P-glycoprotein appears to have a double role, being involved both in protecting cells from drugs and in developing resistance to them. In this context, P-glycoprotein has a function to eliminate given corticosteroids from cytoplasm. These results in inducing corticosteroid resistance, especially in patients with long frequent courses of steroids.⁷

As corticosteroids have been the mainstay of therapy for nephrotic syndrome for nearly 50 years,⁸ for children with steroid resistance or dependency, clinicians should consider appropriate treatment as soon as possible instead of extending therapy with glucocorticoid with its many side effects.⁹ As prognosis of the disease will be determined by appropriate and timely treatment to control disease,¹⁰ certain indicators can be used to determine or diagnose steroid resistance for early introduction of cytotoxic drugs to overcome the mechanism of steroid resistance.

The binding of IL-2 to the high-affinity IL2R may render it immeasurable. Hence, measuring sIL2R is more sensitive.¹¹ Measuring expression levels of *MDR1* mRNA that encodes P-glycoprotein in the peripheral blood nucleated cells using real-time

polymerase chain reaction (PCR) is a more sensitive method than P-glycoprotein protein assay.¹² We aimed in the present study to estimate sIL2R levels and *MDR1* gene expression levels on lymphocytes in NS and to elucidate the relationship between sIL2R and *MDR1* gene expression on lymphocytes in nephrotic patients and their clinical relevance to corticosteroid therapy to determine if any of them can be used as a predictor of steroid resistance. We used sIL2R instead of IL-2 and *MDR1* gene expression instead of P-glycoprotein because they are more sensitive.

MATERIALS AND METHODS

Patients

This is a case-control study followed by longitudinal investigation after our patients entered into the remission phase. We calculated the sample size by calculating the whole population under the study and the prevalence of NS. By using a power estimate of 80% and 95% confidence interval, the sample size was 43 patients. In 3 of the eligible children, their parents refused to participate in the study. Therefore, we studied 40 patients with NS and 20 healthy children as a control group. We obtained informed consent from the parents of the participating children and received approval of our ethical committee. The study was carried during the period from January 2008 to June 2009, applied to the patients in Pediatric Nephrology Unit of the Pediatric Department, Zagazig University Hospitals, in Zagazig, Egypt.

We examined 40 patients with NS (23 boys and 17 girls) with a mean age of 8.3 ± 1.8 years. They were 15 newly diagnosed patients with their first episode of NS and 25 known cases of NS with relapse at the time of study. The latter subgroup was on steroid therapy alone without cytotoxic medication during the past 6 months. We exclude any cases of secondary NS with hepatitis B or C or under any cytotoxic drug rather than corticosteroid. Twenty healthy children (11 boys and 9 girls) with a mean age of 7.7 ± 2.3 years were recruited as a control group.

Diagnosis and Treatment

Nephrotic syndrome was defined as edema, proteinuria greater than 40 mg/m²/h, and hypoalbuminemia. Relapse was diagnosed when proteinuria occurred again (> 40 mg/m²/h) for

3 consecutive days. Remission was determined when urinary protein was less than 4 mg/m²/h for 3 days. Initial response or steroid-sensitive NS (SSNS) was considered if complete remission was achieved within 4 to 6 weeks of steroid therapy. Initial no response or steroid-resistance NS (SRNS) was failure to achieve remission in 6 to 8 weeks with steroid alone.¹³

The patients were treated with prednisone according to an established protocol. At the time of the initial diagnosis, prednisone, 60 mg/m²/d, was started for 4 weeks, and when remission occurred after 4 weeks of daily steroids, the children were tapered to 40 mg/m² on alternate days for an additional 4 weeks and then tapered off steroids over the next 4 to 8 weeks (steroid sensitive). If remission was not attained by 4 weeks, therapy was continued at the same daily dose for up to 8 weeks with addition of cyclophosphamide (steroid resistant). Treatment of relapses was done with oral prednisone, 60 mg/m²/d, until 3 days free of proteinuria was reported. Prednisone was then transitioned immediately to alternate-day therapy, and the dose weaned over 4 to 8 weeks thereafter very gradually according to their clinical response to steroids.¹³

Assessments

All of the patients were examined twice: at baseline (*activity* of the disease) and within 1 week of *remission*. All of the participants were subjected to complete history taking, thorough clinical examination, routine investigations (24-hour urinary protein, serum albumin, complete blood count with differential white blood cell count, serum urea, and serum creatinine). In addition, sIL2R levels and *MDR1* gene expression levels were determined at baseline.

Serum sIL2R. It was measured using an enzyme-linked immunosorbent assay (Cellfree, T Cell Sciences, Cambridge, Massachusetts, USA). In all of the patients, paired samples were taken during early relapse, prior to treatment, and during remission. The serum was stored at 70°C until the assay was performed. Briefly, the principles of the method were as follows: a routine monoclonal antibody to human IL2R was precoated onto polystyrene microtitre wells. Standards and serum samples were added to the wells followed by the addition of a horseradish peroxidase-conjugated anti-IL2R

monoclonal antibody. The sIL2R in the serum binds to the coated antibody while the conjugated antibody binds to a second distinct epitope on the IL2R molecule. Unbound components were removed by washing. A chromagen solution was added to the wells, and the reaction was terminated by addition of sulphuric acid and absorbance read at 490 nm. A standard curve was prepared from the IL2R standards supplied with the kit and unknown values determined from the standard curve. The sensitivity of the assay was 50 U/mL. The reference range of sIL2R was considered 100 U/mL to 500 U/mL.

Expression of *MDR1*. Detection and quantification of *MDR1* gene expression was done on lymphocytes by real-time PCR, using RoboGene MDR-1/GAPDH Quantification Module (Roboscreen, Leipzig, Germany). Real-time PCR instrument was a Smart Cycler (Cepheid, Sunnyvale, CA, USA). Purification of RNA was done for isolation of total RNA from up to 1.5 mL of whole blood, using an Invisorb spinblood RNA Mini Kit (Roboscreen, Leipzig, Germany). A 5-mL sample of venous blood was collected under complete aseptic conditions in sterile ethylenediaminetetraacetic acid Vacutainer.

Protocol of RNA extraction was as follows: (1) Lysis of erythrocytes: 10 mL of cold (4°C) buffer EL was added to 1.0 mL to 1.5 mL of whole blood in a tube (eg, 15-mL Falcon tubes), mixed shortly by vortex, incubated on ice for 15 minutes, and mixed shortly by vortex 2 times during incubation. (2) Collection of leucocytes: Centrifugation at 4000 rpm for 5 minutes was done at 4°C. Then, the supernatant was completely removed (leucocytes will form a visible cell pellet). Five milliliter of Buffer EL was added to the cell pellet, mixed by vortex shortly, and centrifuged again at 4000 rpm for 5 minutes at 4°C. The supernatant was removed as complete as possible. Traces of supernatant have an influence on the further purification process. (3) Lysis of leucocytes: A 900-μL Lysis solution DCT was added to cell pellet and vortex several times to complete removal of any clumps of cells. Then, the cell lysis suspension was transferred into a 2-mL reaction tube (Incubation for 10 minutes). Mixing by vortex was done several times during incubation. (4) Removing of genomic DNA: Centrifugation of the sample was performed at 12 000 rpm for 30 seconds (a white pellet will be

visible). Then, the supernatant was transferred into a new 2-mL tube; carryover of carrier particle was avoided. (5) Binding of the total RNA to the Spin filter: 800 μ L of 70% ethanol was added (must be prepared before) to the supernatant. Mixing of the suspension was done by pipetting several times. Then, 850 μ L of the lysate was transferred into a Spin filter placed in a new 2-mL receiver tube. Centrifugation at 10 000 rpm was carried out for 1 minute. The flow-through was discarded and the Spin filter was placed back into the receiver tube. The residual sample was transferred into the same Spin filter and centrifuged again at 10 000 rpm for 1 minute. The flow-through was discarded and the Spin filter was placed back into the receiver tube. (6) First washing of the Spin filter: 500 μ L of wash buffer R1 was added onto the Spin filter and centrifuged for 30 seconds at 10 000 rpm. The flow-through was discarded and the receiver tube was reused. (7) Second washing of the Spin filter: 750 μ L of wash buffer R2 was added onto the Spin filter, and then centrifuged for 30 seconds at 10 000 rpm. The flow-through was discarded and the receiver tube was reused. This washing step was repeated once again. (8) Drying the Spin filter: To eliminate any traces of ethanol, centrifugation was performed for 3 minutes at 12 000 rpm. The receiver tube was discarded. (9) Elution of total RNA: the Spin filter was transferred into a RNase-free Elution tube. Thirty to 60 μ L of Elution buffer was directly added onto the membrane of the Spin filter. Incubation was done for 2 minutes, and centrifugation for 1 minute at 8000 rpm. Lastly, the Spin filter was discarded and the eluted total RNA was immediately placed on ice.

Reverse transcription for cDNA synthesis from whole RNA was done by reverse transcriptase reaction (The final volume is 25 μ L). Quantification of *MDR1* gene was done by real-time quantitative fluorescence PCR using *MDR1*/*GAPDH* cDNA quantification Module (Roboscreen, Leipzig, Germany).

This assay exploits the so-called *TaqMan* principle. During PCR, forward and reverse primers hybridize to a specific sequence product. A *TaqMan* probe, which is contained in the same reaction mixture and which consists of an oligonucleotide labeled with a 5'-reporter dye and a downstream, 3'-quencher dye, hybridizes to a target sequence within the PCR product. A *Taq* polymerase which

possesses 5'-to-3' exonuclease activity cleaves the probe. The reporter dye and the quencher dye are separated upon cleavage, resulting in an increase in fluorescence for the reporter. Thus, the increase in fluorescence is directly proportional to the target amplification during PCR.

In order to always confirm the RNA integrity of samples as well as to correct for RNA load, cDNA synthesis efficiency, PCR inhibitors, and possible analyte loss during long-time storage it is important to normalize the *MDR1* data to the number of *GAPDH* transcripts measured in the same cDNA sample. The *GAPDH* gene has been reported to be constantly expressed in several cell line, tissues and heterogeneous tumors. Thus, the calculated ratios of both cDNAs reflect the initial ratios of the mRNA in the sample. In addition, in this kit, the manufacturer's standardized was ready to use control and sample reaction PCR tubes which were either coated with control DNA (Both for *MDR1* and *GAPDH*) and amplification enhancer (control tubes), or just the amplification enhancer (sample tubes).

The threshold cycle was defined as the fractional cycle number at which the reporter fluorescence generated by cleavage of the probe passes a fixed threshold above the baseline. Calibration curves for both *MDR1* (using *MDR1* controls) and *GAPDH* (using *GAPDH* controls) were generated on the basis of the linear relationship between the threshold cycle value and the logarithm of the copy numbers. The absolute copy number of *MDR1* and *GAPDH* in samples was calculated by threshold cycle using each of the calibration curves, and the results were standardized by the ratio of the copy number of *MDR1* to the copy number of *GAPDH*.^{14,15} A cutoff was defined for *MDR1* level by running samples of 20 normal healthy individuals (control group). The levels of *MDR1* were estimated and the cutoff was defined according to the following formula:

$$\text{Cutoff} = \text{mean} + 2 \times \text{standard deviation}$$

The mean level of *MDR1* was 2.8% and the standard deviation was 1.5%. Thus, the cutoff was calculated to be 5.8%. An expression level for *MDR1* less than 5.8% was considered as normal level, while higher levels were considered as elevated.

Statistical Analyses

Statistical analyses were performed using the SPSS software (Statistical Package for the Social

Sciences, version 17.0, SPSS Inc, Chicago, Ill, USA). The quantitative data were presented as mean \pm standard deviation and comparisons between more than two groups were done using the 1-way analysis of variance. A *P* value less than .05 was considered significant.

RESULTS

The children with a recent onset of NS ($n = 15$) were 8 boys and 7 girls with a mean age of 6.8 ± 2.1 years. They had no history of steroid therapy before. This group was followed up until remission and subdivided after into those with SSNS ($n = 12$) and those with SRNS ($n = 3$). Children known to have NS with relapse ($n = 25$) were 15 boys and 10 girls with a mean age of 8.2 ± 1.0 years. They were also subdivided into SSNS ($n = 11$) and SRNS ($n = 14$) groups.

Table 1 shows significantly higher levels of sIL2R and *MDR1* gene expression in the patients group both in activity or remission in comparison with the control group. These levels were also significantly higher in activity phase than in remission in the patients group. The sIL2R levels were 1197.2 ± 183.3 U/mL in activity and 791.0 ± 192.6 U/mL in remission as compared to 449.0 ± 27.3 U/mL in the control group. The *MDR1* gene expression was $8.7 \pm 0.9\%$ in activity and $6.3 \pm 1.4\%$ in remission as compared to $2.8 \pm 0.9\%$ in the control group (Table 1).

Table 2 shows the relatively higher levels of both sIL2R and *MDR1* gene expression in relapse

cases than in new onsets either in activity or in remission. Also it shows relatively higher levels of both markers in SRNS cases over SSNS ones either in activity or in remission whether they were new onset or relapse cases. In addition, both markers were relatively higher in the children with relapsed SRNS than in those with new-onset SRNS. Figures 1 and 2 depict levels of sIL2R and *MDR1* gene expression in different groups and subgroups.

A significant positive correlation was observed between serum sIL2R and *MDR1* gene expression among the patients and controls (Figure 3).

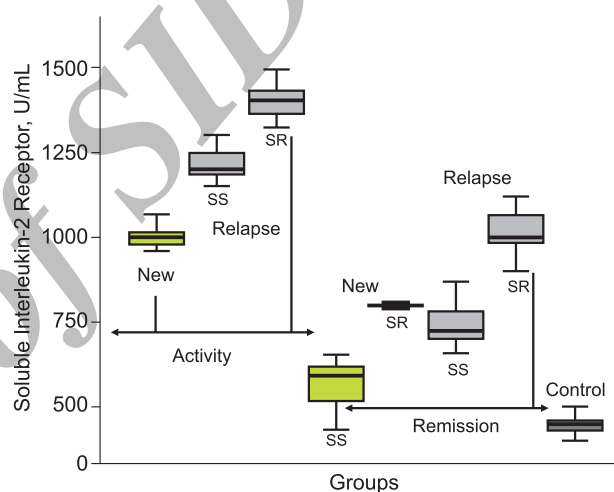


Figure 1. Levels of soluble interleukin-2 receptor in different groups and subgroups. New indicates new onset nephrotic syndrome; Relapse, relapsed nephrotic syndrome; SS, steroid sensitive; and SR, steroid resistant.

Table 1. Mean Serum Albumin, Soluble Interleukin-2 Receptor and *MDR1* Gene Expression Levels in Different Groups

Parameter	Patients in Activity ($n = 40$)	Patients in Remission ($n = 40$)	Controls ($n = 20$)	<i>P</i>
Serum albumin, mg/dL	1.90 ± 0.34	3.00 ± 0.25	3.90 ± 0.39	$< .001$
Soluble interleukin-2 receptor, U/mL	1197.2 ± 183.3	791.0 ± 192.6	449.0 ± 27.3	$< .001$
<i>MDR1</i> gene expression, %	8.7 ± 0.9	6.3 ± 1.4	2.8 ± 0.9	$< .001$

Table 2. Mean Serum Albumin, Soluble Interleukin-2 Receptor and *MDR1* Gene Expression Levels in Different Subgroups

Parameter	Active Disease			Remission				<i>P</i>
	New Onset Patients (n = 15)	Relapse Patients		New Onset Patients		Relapse Patients		
		SS (n = 11)	SR (n = 14)	SS (n = 12)	SR (n = 3)	SS (n = 11)	SR (n = 14)	
Serum albumin, mg/dL	1.92 ± 0.40	1.98 ± 0.40	1.98 ± 0.20	3.00 ± 0.25	2.90 ± 0.15	3.10 ± 0.26	3.00 ± 0.26	< .001
Soluble interleukin-2 receptor, U/mL	992.0 ± 48.6	1216.4 ± 47.8	1401.9 ± 50.8	569.5 ± 65.4	800.3 ± 11.5	745 ± 60.9	1014.8 ± 62.0	< .001
<i>MDR1</i> gene expression, %	7.9 ± 0.4	8.6 ± 0.3	9.7 ± 0.4	4.5 ± 0.5	7.0 ± 0.2	6.0 ± 0.2	7.8 ± 0.6	< .001

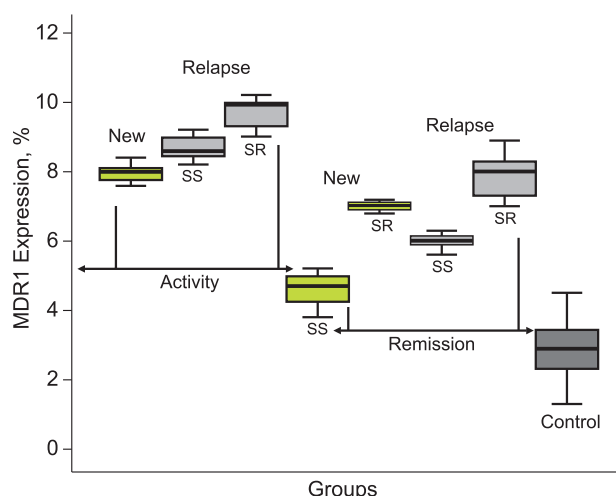


Figure 2. Levels of *MDR1* gene expression in different groups and subgroups. New indicates new onset nephrotic syndrome; Relapse, relapsed nephrotic syndrome; SS, steroid sensitive; and SR, steroid resistant.

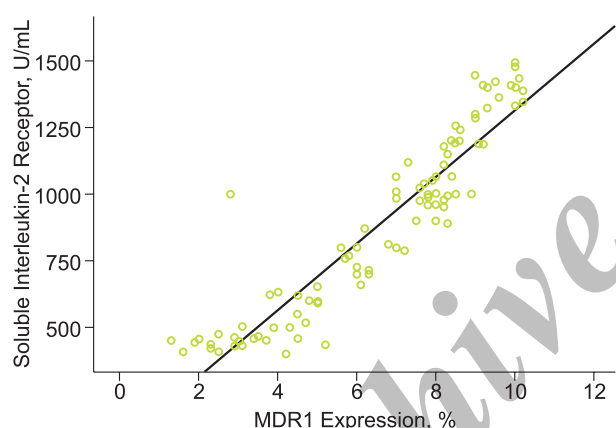


Figure 3. A positive correlation between serum soluble interleukin-2 receptor levels and *MDR1* gene expression levels.

DISCUSSION

In our study, we found a significant increase in serum sIL2R level and *MDR1* gene expression on lymphocytes in pediatric patients with NS in comparison with the control group both in disease activity and remission. These markers were also significantly higher in activity than in remission. Soluble IL2R level and *MDR1* gene expression were significantly elevated in SRNS patients (particularly relapse cases) than SSNS patients, whether in activity or in remission. One of the possible mechanisms of corticosteroid resistance is the overexpression of *MDR1*, which leads to overexpression of P-glycoprotein on peripheral blood lymphocytes.¹⁶ On the other hand, IL-2 is a key

inflammatory mediator involved in pathogenesis of NS, which also increases the expression of *MDR1* on lymphocytes via activation of the transcription factor Y-box protein-1.¹⁷ Interleukin-2 binds to its receptor on lymphocytes and is released into circulation as sIL2R.³ In our study, there was a significant elevation of sIL2R levels in activity as compared to remission, indicating that sIL2R is involved in the pathogenesis of NS. These findings were also reported by Lama and colleagues¹⁸ who found an elevation of IL-2 in acute phase of the disease.

We also found that sIL2R elevations were more significant in the patients with SRNS than in SSNS ones. This agrees with Aviles and coworkers who reported that higher IL-2 mRNA expression was observed in patients with SRNS than in those with SSNS.¹⁹ Furthermore, sIL2R returned nearly to a normal level in the remission phase of patients with SSNS, comparable to values in the control group. This finding was in agreement with Shimoyama and colleagues' results.³

In steroid-resistant nephrotic patients with their first remission and in all relapsing nephrotic patients, sIL2R was still significantly elevated as compared to controls. As sIL2R is a key inflammatory mediator involved in pathogenesis of NS,²⁰ our data suggest that persistent elevation of sIL2R in new cases with steroid-resistant and relapsing nephrotic patients indicates that the lymphocytes activity is not completely suppressed under the effect of steroid therapy, and there is ongoing inflammatory process. Soluble IL2R is significantly elevated in steroid-resistant patients than in steroid-sensitive patients, whether in activity or in remission; we hypothesized that the significant elevation of sIL2R in SRNS, whether in relapse or remission, favors the theory that sIL2R is involved in the mechanism of steroid resistance as high levels of sIL2R are associated with poor response to steroids, and this was in agreement with Swiatecka and associates.²¹

In our study, *MDR1* expression on lymphocytes was significantly elevated in nephrotic patients in activity and remission compared to normal healthy controls, and these elevations were more significant in activity than in remission. This finding is in agreement with Funki and colleagues⁴ who found that *MDR1* gene expression is high in the first attack or in relapse compared with those in

remission. This may explain why patients in the first attack or in relapse require higher doses of corticosteroids than in remission to obtain the same drug action.

In our study, during the disease activity, *MDR1* expression is significantly elevated in relapsing nephrotic patients than nephrotic patients in their first episode, and these elevations were more significant in steroid-resistant relapsing nephrotic patients than other subgroups; thus, we hypothesized that nephrotic patients who are exposed to long and frequent courses of steroids have the highest *MDR1* expressions on lymphocytes. This is in agreement with Maillefert and coworkers²² who found P-glycoprotein surface overexpression in peripheral lymphocytes from relapsing patients under long-term steroid therapy. P-glycoprotein a substrate of *MDR1* gene that captures drugs when they pass through the cell membrane and then releases them outside the cell, when the number of P-glycoprotein molecules expressed on lymphocytes is high, corticosteroids, which are the substrate of P-glycoprotein, are not able to reach the cytoplasm. In this situation, the responsiveness to steroids becomes worse. Thus, we hypothesized that in our study, the high expression of *MDR1*, especially in steroid-resistant frequent-relapsing nephrotic patients who are under long-term steroid therapy, may be responsible for the mechanism of steroid resistance. These data are supported by finding of higher levels of *MDR1* expression in poor responders to steroids, this agrees with Tsujimura and coworkers.⁵

In this current study, there was a positive correlation between *MDR1* gene expression levels and *sIL2R* levels, meaning that *sIL2R*, which is involved not only in NS pathogenesis, but also in SRNS, upregulated *MDR1* gene expressions on lymphocytes. The upregulated *MDR1* gene, especially in steroid-resistant frequent-relapsing nephrotic patients with long frequent courses of steroids, causes ineffective steroid response; thus, the immunological dysregulation in nephrotic patients is not controlled under the effect of corticosteroids alone with poor prognosis of disease.

CONCLUSIONS

Our study propose using *sIL2R* and/or *MDR1* gene expression levels as early predictors of steroid resistance in nephrotic patients, in order to

promote proper early control of disease by proper early introduction of cytotoxic drugs to overcome the effect of P-glycoprotein as an efflux pump of steroids, and hence, saving patients from long periods of exposure to steroids without benefit. As this is the first report providing new insight into the use of *sIL2R* as a predictor of steroid resistance, a wide-scale study is needed to determine a cutoff level of *sIL2R* above which cytotoxic drugs are introduced early in course of the disease to suppress activated lymphocytes with subsequent decrease in *sIL2R* and *MDR1* gene expression.

CONFLICT OF INTEREST

None declared.

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