Markers of disease and steroid responsiveness in paediatric idiopathic nephrotic syndrome: Whole-transcriptome sequencing of peripheral blood mononuclear cells

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Abstract
Objective: To identify markers of disease and steroid responsiveness in paediatric idiopathic nephrotic syndrome.
Methods: Whole-transcriptome sequencing was performed of peripheral blood mononuclear cells (PBMCs) from patients with NS. Differentially expressed genes (DEGs) were identified in patients with active NS vs those in remission, and those with steroid-sensitive NS (SSNS) vs steroid-resistant NS (SRNS).
**Results:** A total of 1065 DEGs were identified in patients with NS \((n = 10)\) vs those in remission \((n = 9)\). These DEGs correlated with cytokine and/or immune system signalling and the extracellular matrix. Comparisons between SSNS \((n = 6)\) and SRNS \((n = 4)\) identified 1890 DEGs. These markers of steroid responsiveness were enriched with genes related to the cell cycle, targets of microRNAs, and genes related to cytokines.

**Conclusions:** Meaningful DEGs were identified. Additional studies with larger numbers of patients will provide more comprehensive data.

**Keywords**
Nephrotic syndrome, signature, transcriptome

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**Introduction**

The first-line therapy for children with idiopathic nephrotic syndrome (NS) is steroid treatment, which induces remission in most patients.\(^1\)\(^-\)\(^3\) The main clinical problems associated with steroid-sensitive NS (SSNS) are frequent relapse and subsequent drug toxicity.\(^4\) Patients with steroid-resistant NS (SRNS) who do not respond to steroids and other treatments are at risk of the deterioration of renal function leading to end-stage renal disease.\(^5\)\(^,\)\(^6\) Both SSNS and SRNS are associated with effacement of glomerular epithelial cell (podocyte) foot processes, a cardinal morphological feature of NS.\(^7\) The aetiology of podocytopathy resulting in NS, reasons for steroid non-responsiveness, and the mechanisms underlying relapse in SSNS remain to be fully established.\(^8\)

It has been speculated that the pathophysiology of SSNS involves disturbance of the immune system, especially T cells. This speculation is based on findings including the association between NS and lymphoma in some cases, relapse coinciding with infection, response to various immunosuppressive medications, and imbalances of a subpopulation of lymphocytes.\(^3\)\(^,\)\(^9\)\(^-\)\(^12\) Several cytokines and other soluble plasma components may also be associated with NS.\(^13\)\(^,\)\(^14\) A case has been described in which SSNS disappeared after bone-marrow transplantation,\(^10\) suggesting that hematopoietic cells are involved in the pathogenesis of SSNS. SRNS has been shown to recur after kidney transplantation in some patients, suggesting that the pathogenesis of this condition resides outside the kidney; in addition, the efficacy of plasmapheresis in most recurrent cases indicates the presence of circulating factor(s) that cause SRNS.\(^15\)\(^,\)\(^16\) However, these contributing factors remain to be identified and validated.\(^17\)\(^-\)\(^20\) Although immunosuppressive agents are effective in some patients with SRNS, there are currently no tools to determine the optimal treatment for a patient before a therapeutic trial, or for predicting recurrence after kidney transplantation.\(^15\)\(^,\)\(^21\)

Comprehensive information regarding NS would lead to a better understanding of the pathogenesis of the disease, mechanism of relapse, optimal medication choice, and prediction of prognosis. Thus, the present study applied whole-transcriptome sequencing of peripheral blood mononuclear cells (PBMCs) from patients with NS, using a next-generation sequencing (NGS) method of RNA sequencing.\(^22\) PBMCs were used because of the high probability of immune system involvement in the pathogenesis of NS, and their easy accessibility, a prerequisite for a useful biomarker.\(^23\)\(^,\)\(^24\) Compared with microarray
technologies, RNA sequencing can capture the dynamic range of transcriptomes in terms of both expression profiling and differentially expressed isoforms (DEIs) on a massive scale.\textsuperscript{25–27} We report the preliminary results of signature gene sets of NS and steroid responsiveness.

**Patients and methods**

**Study population**

The study recruited children aged <18 years who were newly diagnosed with idiopathic NS at Seoul National University Children’s Hospital, Seoul, Republic of Korea, between January 2008 and December 2011. Patients who were on long-term treatment prior to transfer to our hospital were excluded from the study. Pathological diagnosis was obtained only in patients with SRNS.

The study was approved by the Seoul National University Hospital Institutional Review Board (No. 0812-002-264), and the participants’ parents or legal guardians provided written informed consent prior to enrolment.

**Sample collection**

Peripheral blood samples were collected from patients and PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation, then stored at −80°C until RNA extraction. Nephrotic samples were collected at the time of onset or relapse of NS, before commencing any treatment. Remission samples were collected from patients with SSNS during remission, when having not been taking steroids for >2 months.

**Whole-transcriptome sequencing**

Total RNA was extracted from PBMCs using a QIAamp RNA mini kit (Qiagen, Austin, TX, USA). Libraries were prepared based on the Illumina protocol according to the manufacturer’s instructions, and 54 bp of paired-end RNA sequencing data were generated using the Illumina Genome Analyzer IIx (Illumina, San Diego, CA, USA). The prepared libraries were quantified using quantitative polymerase chain reaction (PCR) according to the quantification protocol guide in the manufacturer’s instructions. The read quality was checked, then the differentially expressed gene (DEG) sequences were identified using R package DEGseq (version 1.10.0),\textsuperscript{28} through counting the reads and assessing the distribution of count differences between samples. Raw read quality scores and read counts were summarized.

For annotation, RNA sequence reads were aligned to the human reference genome (University of California, Santa Cruz [UCSC] hg19; 20 October 2011) using TopHat software (version 1.4.0)\textsuperscript{30} and Bowtie software (version 1.12.5),\textsuperscript{31} with the supplied annotations, a set of gene-model annotations and known transcripts, and the \texttt{–no-novel-juncs} option to disable mapping for novel splice junctions.\textsuperscript{29–31} The aligned reads were quantified with Cufflinks (version 1.3.0) to obtain the fragments per kilobase of exons per million fragments mapped (FPKM) values for the genes or gene transcripts, and then merged into an expression table for the next analysis step, outlined in Figure 1 and conducted as described.\textsuperscript{31}

**Expression profiling and functional annotation**

The average number of reads produced from each sample was 74 million. Only those of protein coding genes listed on the UCSC Genome Browser\textsuperscript{32} were analysed. Loci with low variance in FPKM values or zero reads across all samples were removed. Variance-stabilizing normalization and upper-quartile normalization were applied to the boost sensitivity without a loss of specificity.\textsuperscript{33}

The DEGs were obtained from one-way analyses of variance (ANOVA) for
each group, and false discovery rate (FDR) multiple testing corrections were applied. Post-hoc analyses were performed to detect the relationships between groups via the Tukey’s honest significance test. Analyses of DEIs were performed similarly, but no significant DEIs were obtained.

The DEGs of the groups of interest were obtained by t-tests. For functional annotation and clustering, the Gene Set Enrichment Analysis (GSEA) program (version 2.0.8) with the Molecular Signatures Database (version 3.1) and the Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7) were used to enhance understanding of the underlying biological relevance. Clustering analysis was performed using the kmeans function in R 3.0.2, which performs k-means clustering ($K = 10$ clusters specified) on a given expression profile for DEGs. The hypergeometric distribution is used to compute $P$-values for Gene Ontology (GO) annotation for clusters with the Molecular Signatures Database (version 5.1). For upstream analysis of DEGs, gene-sets of microRNA targets ($n = 221$) and transcription factor targets ($n = 615$) from Molecular Signatures Database (version 5.1) were downloaded and compared with DEGs.

Results

In total, 18 patients with idiopathic NS were enrolled (15 males/3 females; mean age $8.2 \pm 4.0$ years; age range 2.7–16.7 years). The median age at onset of NS was 5.9 years (range 3.0–14.4 years). Nephrotic samples
(n = 10) were obtained from six patients with SSNS and four with SRNS. Pathological diagnosis was obtained only in those with SRNS, and was focal segmental glomerulosclerosis in all cases. Of the four patients with SRNS, two responded to cyclosporine treatment (calcineurin inhibitor [CNI] responders [CRs]), and two responded to neither steroids nor CNI (nonresponders [NRs]). A total of nine remission samples were collected from patients with SSNS.

The gene expression profile was determined by analysing 19 samples from 18 patients (one patient provided both a nephrotic sample and a remission sample) and 18 551 genes. Statistical analyses identified 1065 DEGs in the NS group (n = 10) relative to the remission group (n = 9) (Figure 2). Functional annotations of these genes revealed that these DEGs were related to dorsal/ventral pattern formation (enrichment score [ES] 2.05), extracellular matrix structural constituents (ES 1.75), and actin binding (ES 1.36) according to the DAVID functional annotation module. Based on the GSEA, compared with the remission group, the gene-expression profile of the NS group was enriched with genes pertaining to steroid hormones, matrix metalloproteinase (i.e., enzymes that degrade the extracellular matrix)-inducing cytokines, extracellular matrix-receptor interaction, acyl chain remodelling of phosphatidylglycerol, G βγ signalling through PI3Kγ, CTLA4 inhibitory signalling, the early response to TGFβ1, IL4 receptor signalling in B lymphocytes, pantothenate and CoA biosyntheses, the syndecan 3 pathway, and the mTOR signalling pathway.

More stringent criteria (P < 0.01 and >2-fold changes of expression) were applied to identify the highly significant genes in idiopathic NS. A total of 49 genes were found to be significantly upregulated in NS, and 67 genes were found to be downregulated (Table 1). K-means clustering for 116 DEGs revealed 10 clusters of 3–37 genes, with enriched GO terms listed in Table 2 (hypergeometric test, P < 0.005). Upstream analysis revealed that DEGs of NS were enriched with targets of MIR-370 (P = 0.0163, reported in Wilms tumour) and MIR-519E (P = 0.0428, clinical relevance not yet known), as well as targets of transcription factors ATF2, ATF6, EVI1, HMGA1, IRF8, ITGAL, JUN, MEF2A, NFAT, PGR, POU3F2, and STAT6.

Gene expression patterns differed significantly between SSNS and SRNS (Figure 3), with 1890 DEGs identified (P < 0.1). These DEGs were enriched with genes related to the microtubule organizing centre and regulation of the response to biotic stimuli based on the GO terms. Based on the GSEA, compared with the SRNS group, the gene expression profile of the SSNS group was enriched with genes pertaining to TGFβ1 signalling, the cell cycle and p53 signalling, Y branching of actin filaments, FoxP3 targets in T lymphocytes, cytokines IL6 and
Table 1. Differentially expressed genes (DEGs) in paediatric idiopathic nephrotic syndrome (NS). (nephrotic status vs remission status; \( P < 0.01 \); relative change >2-fold).

| Gene symbol | Official gene name | Relative change | Statistical significance |
|-------------|-------------------|----------------|-------------------------|
| RWDD1       | RWD domain-containing 1 | 6.3 | \( P = 0.000073 \) |
| IVD         | Isovaleryl coenzyme A dehydrogenase | 2.2 | \( P = 0.00019 \) |
| ZNF48       | Zinc finger protein 48 | 6.6 | \( P = 0.0002 \) |
| FAM65B      | Family with sequence similarity 65, member B | 4.8 | \( P = 0.00038 \) |
| USP2        | Ubiquitin-specific peptidase 2 | 3.0 | \( P = 0.00038 \) |
| DNMT3B      | DNA (cytosine-5-)methyltransferase 3 beta | 3.4 | \( P = 0.00038 \) |
| DUSP23      | Dual specificity phosphatase 23 | 4.6 | \( P = 0.00110 \) |
| ZNF229      | Zinc finger protein 229 | 3.9 | \( P = 0.0012 \) |
| C11orf74    | Chromosome 11 open reading frame 74 | 2.1 | \( P = 0.0014 \) |
| CAPN6       | Calpain 6 | 2.0 | \( P = 0.0018 \) |
| LARP6       | La ribonucleoprotein domain family, member 6 | 3.7 | \( P = 0.0018 \) |
| BIRC6       | Baculoviral IAP repeat containing 6 | 2.2 | \( P = 0.0019 \) |
| TMEM134     | Transmembrane protein 134 | 3.9 | \( P = 0.0019 \) |
| RPS15A      | Ribosomal protein S15a pseudogene 17 | 2.3 | \( P = 0.0021 \) |
| ETV4        | ETS translocation variant 4 | 5.6 | \( P = 0.0022 \) |
| SLFN5       | Schlafen family member 5 | 2.2 | \( P = 0.0023 \) |
| ROR2        | Receptor tyrosine kinase-like orphan receptor 2 | 6.5 | \( P = 0.0024 \) |
| GADD45G     | Growth arrest and DNA-damage-inducible, gamma | 7.8 | \( P = 0.0025 \) |
| HAUS4       | HAUS augmin-like complex, subunit 4 | 2.3 | \( P = 0.0025 \) |
| SNTA1       | Syntrophin, alpha 1 (dystrophin-associated protein A1, 59-kDa, acidic component) | 2.2 | \( P = 0.003 \) |
| SIRT6       | Sirtuin (silent mating type information regulation 2 homologue) 6 (Saccharomyces cerevisiae) | 2.4 | \( P = 0.0037 \) |
| CD8A        | CD8a molecule | 3.6 | \( P = 0.0038 \) |
| OTP2        | Otopetrin 2 | 2.6 | \( P = 0.0038 \) |
| AMIGO1      | Adhesion molecule with Ig-like domain 1 | 2.4 | \( P = 0.004 \) |
| C15orf48    | Chromosome 15 open reading frame 48 | 2.1 | \( P = 0.0041 \) |
| MPP6        | Membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6) | 5.4 | \( P = 0.0044 \) |

(continued)
| Gene symbol | Official gene name                                                                 | Relative change | Statistical significance |
|-------------|------------------------------------------------------------------------------------|-----------------|--------------------------|
| EPT1        | Selenoprotein 1                                                                     | 3.7             | $P = 0.0046$             |
| TTLL12      | Tubulin tyrosine ligase-like family, member 12                                      | 3.5             | $P = 0.0048$             |
| WDR5        | WD-repeat domain 5                                                                   | 2.3             | $P = 0.0049$             |
| IL1RAP      | Interleukin 1 receptor accessory protein                                             | 2.2             | $P = 0.0053$             |
| WDR27       | WD-repeat domain 27                                                                  | 2.4             | $P = 0.0054$             |
| CYB5B       | Cytochrome b5 type B (outer mitochondrial membrane)                                  | 2.9             | $P = 0.0054$             |
| PTK2        | PTK2 protein tyrosine kinase 2                                                       | 4.3             | $P = 0.0055$             |
| LAMA4       | Laminin, alpha 4                                                                    | 3.7             | $P = 0.0055$             |
| SGK223      | Homologue of rat pragma of Rnd2                                                      | 2.3             | $P = 0.0058$             |
| DAZAP2      | DAZ-associated protein 2                                                             | 2.0             | $P = 0.0058$             |
| LAX1        | Lymphocyte transmembrane adaptor 1                                                  | 4.3             | $P = 0.0059$             |
| C17orf100   | Chromosome 17 open reading frame 100                                                 | 2.0             | $P = 0.006$              |
| PEBP1       | Phosphatidylethanolamine-binding protein 1                                           | 3.8             | $P = 0.006$              |
| BAI1        | Brain-specific angiogenesis inhibitor 1                                              | 5.6             | $P = 0.0062$             |
| RMND5A      | Required for meiotic nuclear division 5 homologue A (S. cerevisiae)                  | 4.6             | $P = 0.0064$             |
| OSCP1       | Chromosome 1 open reading frame 102                                                  | 3.2             | $P = 0.0065$             |
| WNT5A       | Wingless-type MMTV integration site family, member 5A                                 | 9.9             | $P = 0.0067$             |
| LMX1B       | LIM homeobox transcription factor 1, beta                                            | 2.3             | $P = 0.0075$             |
| TYMP        | Thymidine phosphorylase                                                              | 4.6             | $P = 0.0075$             |
| HIST1H2BN   | Histone cluster 1, H2bn                                                             | 2.8             | $P = 0.0078$             |
| MYH2        | Myosin, heavy chain 2, skeletal muscle, adult                                        | 2.8             | $P = 0.0087$             |
| IFN5        | Interferon, alpha 5                                                                 | 4.3             | $P = 0.0096$             |
| MMP24       | Matrix metallopeptidase 24 (membrane-inserted)                                       | 2.4             | $P = 0.0099$             |
| NDST2       | N-deacetylase/N-sulfotransferase (heparanglucosaminy) 2                              | 2.1             | $P = 0.00332$            |
| ZNF670      | Zinc finger protein 670                                                              | 2.1             | $P = 0.0045$             |
| VNN1        | Vanin 1                                                                             | 2.1             | $P = 0.00231$            |
| CTIF        | CBP80/20-dependent translation initiation factor                                      | 2.2             | $P = 0.000469$           |
| NEDD1       | Neural precursor cell expressed, developmentally down-regulated 1                   | 2.2             | $P = 0.00907$            |
| ALKBH8      | AlkB, alkylatation repair homolog 8 (Escherichia coli)                               | 2.3             | $P = 0.00594$            |
| CDKN3       | Cyclin-dependent kinase inhibitor 3                                                  | 2.3             | $P = 0.00890$            |

(continued)
| Gene symbol | Official gene name                                                                 | Relative change | Statistical significance |
|-------------|-----------------------------------------------------------------------------------|-----------------|----------------------------|
| TNNC2       | Troponin C type 2 (fast)                                                          | −2.3            | \( P = 0.00225 \)         |
| IL2I        | Interleukin 21                                                                    | −2.4            | \( P = 0.00711 \)         |
| LCN2        | Lipocalin 2                                                                       | −2.4            | \( P = 0.0055 \)          |
| PEL13       | Pellino homologue 3 (Drosophila)                                                   | −2.4            | \( P = 0.00426 \)         |
| DIRAS3      | DIRAS family, GTP-binding RAS-like 3                                               | −2.5            | \( P = 0.00238 \)         |
| PRKD2       | Protein kinase D2                                                                  | −2.5            | \( P = 0.00909 \)         |
| SLC26A9     | Solute-carrier family 26, member 9                                                 | −2.7            | \( P = 0.00517 \)         |
| AGXT2 L1    | Alanine-glyoxylate aminotransferase 2-like 1                                      | −2.8            | \( P = 0.00712 \)         |
| TMEM81      | Transmembrane protein 81                                                           | −2.8            | \( P = 0.0023 \)          |
| REEP2       | Receptor accessory protein 2                                                       | −3.0            | \( P = 0.00516 \)         |
| WNT8B       | Wingless-type MMTV integration site family, member 8B                              | −3.1            | \( P = 0.00381 \)         |
| SPZ1        | Spermatogenic leucine zipper 1                                                     | −3.1            | \( P = 0.00892 \)         |
| HLC5        | Holocarboxylasesynthetase (biotin-[propionyl-coenzyme A-carboxylase (ATP-hydrolysing)] ligase) | −3.2            | \( P = 0.0000898 \)      |
| SGK2        | Serum/glucocorticoid-regulated kinase 2                                            | −3.3            | \( P = 0.00829 \)         |
| SLC10A6     | Solute carrier family 10 (sodium/bile acid cotransporter family), member 6        | −3.4            | \( P = 0.00696 \)         |
| ERV-V-2     | Endogenous retrovirus group V, member 2                                            | −3.4            | \( P = 0.00302 \)         |
| OPLAH       | 5-Oxoprolinase (ATP-hydrolysing)                                                   | −3.4            | \( P = 0.00323 \)         |
| PXMP4       | Peroxisomal membrane protein 4, 24 kDa                                             | −3.5            | \( P = 0.00689 \)         |
| ANO10       | Anocytin 10                                                                       | −3.5            | \( P = 0.00627 \)         |
| ST8SIA1     | ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1                       | −3.6            | \( P = 0.00548 \)         |
| RAD51AP2    | RAD51-associated protein 2                                                         | −3.6            | \( P = 0.00591 \)         |
| PRPF38B     | PRPF38 pre-mRNA processing factor 38 (yeast) domain containing B                  | −3.8            | \( P = 0.00388 \)         |
| RPFL4B      | Ret finger protein-like 4B                                                         | −4.0            | \( P = 0.007 \)           |
| PSRC1       | Proline-serine-rich coiled-coil 1                                                  | −4.1            | \( P = 0.00206 \)         |
| FAM55C      | Family with sequence similarity 55, member C                                       | −4.5            | \( P = 0.00554 \)         |
| TRIML2      | Tripartite motif family-like 2                                                     | −4.5            | \( P = 0.00399 \)         |
| MATN1       | Matrilin 1, cartilage matrix protein                                               | −4.5            | \( P = 0.00207 \)         |
| MED29       | Mediator complex subunit 29                                                        | −4.6            | \( P = 0.00543 \)         |
| STH         | Saitohin                                                                          | −4.6            | \( P = 0.00238 \)         |

(continued)
| Gene symbol | Official gene name                                                                 | Relative change | Statistical significance |
|-------------|-----------------------------------------------------------------------------------|-----------------|--------------------------|
| CCDC64      | Coiled-coil domain containing 64                                                  | -4.6            | P = 0.00665              |
| KIAA0922    | KIAA0922                                                                          | -4.9            | P = 0.00479              |
| LY75        | CD302 molecule; lymphocyte antigen 75                                              | -4.9            | P = 0.00985              |
| CYP11B1     | Cytochrome P450, family 11, subfamily B, polypeptide 1                             | -5.3            | P = 0.00456              |
| TSC2        | Tuberous sclerosis 2                                                              | -5.6            | P = 0.00627              |
| CASQ2       | Calsequestrin 2 (cardiac muscle)                                                   | -5.7            | P = 0.00237              |
| MAGEA9      | Melanoma antigen family A, 9; melanoma antigen family A, 9B                       | -5.8            | P = 0.00104              |
| ZNF358      | Zinc finger protein 358                                                           | -5.9            | P = 0.00897              |
| TBX20       | T-box 20                                                                           | -6.1            | P = 0.00765              |
| CYP2C9      | Cytochrome P450, family 2, subfamily C, polypeptide 9                             | -6.3            | P = 0.00386              |
| FYN         | FYN oncogene related to SRC, FGR, YES                                              | -6.3            | P = 0.00701              |
| LRR1Q4      | Leucine-rich repeats and IQ motif-containing 4                                     | -6.4            | P = 0.00827              |
| CLOCK       | Clock homolog (mouse)                                                             | -6.6            | P = 0.00433              |
| SLC7A10     | Solute carrier family 7 (neutral amino acid transporter, y + system), member 10   | -6.8            | P = 0.00315              |
| HOXA11      | Homeobox A11                                                                       | -7.0            | P = 0.00935              |
| SPINT4      | Serine peptidase inhibitor, Kunitz type 4                                          | -7.1            | P = 0.00225              |
| PAGE4       | P antigen family, member 4 (prostate-associated)                                   | -7.2            | P = 0.00475              |
| OPTC        | Opticin                                                                           | -7.7            | P = 0.00442              |
| CEACAM20    | Carcinoembryonic antigen-related cell adhesion molecule 20                        | -7.8            | P = 0.00209              |
| ZFHX2       | Zinc finger homeobox 2                                                             | -8.1            | P = 0.0068               |
| CHRD        | Chordin                                                                           | -8.5            | P = 0.00949              |
| TGM7        | Transglutaminase 7                                                                | -8.5            | P = 0.00491              |
| CHIA        | Chitinase, acidic                                                                  | -8.9            | P = 0.00159              |
| SVIP        | Small VCP/p97-interacting protein                                                  | -9.0            | P = 0.00863              |
| AOX1        | Aldehyde oxidase 1                                                                 | -9.5            | P = 0.00905              |
| FAM75A6     | Family with sequence similarity 75, member A6                                      | -9.7            | P = 0.00053              |
| LIPI        | Lipase, member 1                                                                   | -10.1           | P = 0.00344              |
| KCNA10      | Potassium voltage-gated channel, shaker-related subfamily, member 10              | -10.7           | P = 0.00366              |
| SLC34A2     | Solute carrier family 34 (sodium phosphate), member 2                              | -11.4           | P = 0.0048               |
| LRP18       | Low-density lipoprotein-related protein 1B (deleted in tumours)                    | -11.6           | P = 0.00936              |
| S100A9      | S100 calcium-binding protein A9                                                    | -21.0           | P = 0.000391             |
Table 2. Enriched Gene Ontology (GO) terms from K-means clustering of differentially expressed genes (DEGs) in paediatric idiopathic nephrotic syndrome (NS). Clusters listed based on hypergeometric test of $P < 0.005$.

| Cluster | Genes | Enriched GO term(s) | Statistical significance |
|---------|-------|---------------------|-------------------------|
| Cluster 1, $n = 4$ | AMIGO1, CYB5B, HIST1H2BN, MMP24 | Heterophilic cell adhesion | $P = 0.0022$ |
| | | Homophilic cell adhesion | $P = 0.0034$ |
| | | Regulation of action potential | $P = 0.0037$ |
| | | Cell recognition | $P = 0.0041$ |
| Cluster 2, $n = 37$ | ALKBH8, CCDC64, CDKN3, DAZAP2, DUSP23, EPT1, FAM65B, HAUS4, HLC5, IVD, LCN2, LMX1B, MATN1, MMP6, NDST2, NEDD1, OPLAH, OPTC, OSCP1, OTOP2, PEL13, PSRC1, PXMP4, REEP2, RPS15A, SIRT6, SLFN5, SNTA1, ST8SIA1, TMEM81, TNNC2, TYMP, VNN1, WNT8B, ZFHX2, ZNF48, ZNF670 | Transferase activity transferring pentosyl groups | $P = 0.0007$ |
| | | Extracellular matrix structural constituent | $P = 0.0013$ |
| Cluster 3, $n = 4$ | CASQ2, FAM75A6, MED29, STH | Striated muscle contraction | $P = 0.0030$ |
| Cluster 4, $n = 23$ | ANO10, BAI1, BIRC6, C1orf100, CAPN6, CTIF, DNMT3B, ETV4, FAM55C, GADD45G, IFNAS5, ILIRAP, LARP6, LAX1, MYH2, PEBP1, PTK2, RMNDS5A, ROR2, SGK223, TMEM134, USP2, WDR27 | SH2 domain binding | $P = 0.0002$ |
| | | Cysteine type endopeptidase activity | $P = 0.0011$ |
| Cluster 7, $n = 5$ | AOX1, CHIA, CHRD, CLOCK, LY75 | Inflammatory response | $P = 0.0005$ |
| | | N acetylglycosaminemtabolic process | $P = 0.0032$ |
| Cluster 8, $n = 9$ | CEACAM20, FYN, LAMA4, S100A9, SLC7A10, SPZ1, SVIP, TGM7, TSC2 | Neutral amino acid transport | $P = 0.0048$ |
IL4, and targets of MIR106B (related to renal cell carcinoma) and MIR16 (expressed in the kidneys).

More stringent criteria (\(P < 0.01\) and 2-fold changes of expression) were applied to identify the markers of steroid responsiveness. Consequently, 23 genes were selected (Table 3; enriched GO terms per k-means clustering Table 4). Upstream analysis did not reveal any significant findings.

**Discussion**

This study used whole-transcriptome sequencing to identify genes that differed in expression in children with idiopathic NS in remission or with nephrotic status. Analysis using \(t\)-testing with \(P < 0.05\) revealed 1065 DEGs for NS independent of steroid responsiveness. These DEGs were enriched with extracellular matrix structural constituent/actin binding/cytoskeletal protein binding according to the GO term of molecular function, as well as cytokine and/or immune system signalling related to steroids; CTLA4, TGF\(\beta\)1, IL4, and mTOR according to GSEA. IL4 is a representative cytokine of Th2 immune reactions, and Th2 immune reactions have been reported to be predominantly associated with childhood NS. Additionally, CTLA4 and TGF\(\beta\)1 are related to immune regulation, and impaired regulatory T cell function has been reported in idiopathic NS. Upstream analysis showed that DEGs of NS were enriched with targets of MIR-370, which is related to Wilms tumour of the kidneys, suggesting relevance of DEGs affecting the kidneys. Furthermore, among 12 upstream genes, ITGAL, MEF2A, STAT6 are members of steroid responsiveness panel genes in U.S. patents. Therefore, the findings of the present study generally agree with knowledge regarding NS. Further refinement of these results in larger studies will improve our understanding of NS.
Table 3. Differentially expressed genes (DEGs) in paediatric patients with steroid sensitive idiopathic nephrotic syndrome (SSNS) or steroid resistant nephrotic syndrome (SRNS) ($P < 0.01$; relative change $>2$-fold).

| Gene symbol | Official gene name                                                                 | Relative change | Statistical significance |
|-------------|-----------------------------------------------------------------------------------|----------------|--------------------------|
| TCF4        | Transcription factor 4                                                             | 11.3           | $P = 0.0067$             |
| BMPR1B      | Bone morphogenetic protein receptor, type IB                                      | 8.5            | $P = 0.008$              |
| LOC255411   | Hypothetical LOC255411                                                             | 6.7            | $P = 0.0075$             |
| ITGA4       | Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)              | 6.4            | $P = 0.00017$            |
| XKR6        | XK, Kell blood group complex subunit-related family, member 6                      | 6.3            | $P = 0.0041$             |
| PLA2G5      | Phospholipase A2, group V                                                          | 6.1            | $P = 0.00017$            |
| DSG4        | Desmoglein 4                                                                      | 5.5            | $P = 0.004$              |
| ODZ4        | Teneurin transmembrane protein 4                                                  | 5.4            | $P = 0.00011$            |
| GAGE2D      | G antigen 2A                                                                       | 5.2            | $P = 0.004$              |
| KRTAP5-10   | Keratin-associated protein 5-10                                                    | 5.1            | $P = 0.0038$             |
| OC90        | Otoconin 90                                                                       | 4.8            | $P = 0.0074$             |
| GPC1        | Glypican 1                                                                        | 4.7            | $P = 0.0042$             |
| FAM48B2     | Family with sequence similarity 48, member B2                                      | 4.5            | $P = 0.0041$             |
| MSRB3       | Methionine sulphoxide reductase B3                                                 | 4.3            | $P = 0.0065$             |
| ZXDC        | ZXD family zinc finger C                                                            | 2.9            | $P = 0.000052$           |
| ZNF566      | Zinc finger protein 566                                                            | 2.3            | $P = 0.0051$             |
| ZNF251      | Zinc finger protein 251                                                            | -2.0           | $P = 0.00085$            |
| SAP30BP     | SAP30-binding protein                                                              | -2.3           | $P = 0.003$              |
| LGALS4      | Lectin, galactoside-binding, soluble, 4                                            | -2.8           | $P = 0.0042$             |
| RAD9B       | RAD9 homologue B (Schizosaccharomyces pombe)                                       | -3.6           | $P = 0.0012$             |
| ATCAY       | Ataxia, cerebellar, Cayman type                                                    | -3.7           | $P = 0.0049$             |
| PDF         | Peptide deformylase                                                               | -4.0           | $P = 0.0048$             |
Although steroid treatment is the first-line treatment for children with NS, it is associated with significant toxicity.\(^4\) For patients who do not respond to steroid treatment, initial treatment with steroids could be harmful as well as ineffective. Moreover, more aggressive treatments, such as CNI, rituximab and plasmapheresis, could induce remission in many patients if instituted without delay, as seen in recurrent SRNS after kidney transplantation.\(^{15,21}\) Therefore, the identification of reliable markers for steroid responsiveness would allow more directed treatment of paediatric NS. Patients who are nonresponsive to steroids could be other treatment options without delay. In search of markers for steroid responsiveness in paediatric NS, we identified a total of 1890 DEGs, and selected 23 genes based on more stringent criteria. Interestingly, the DEGs of patients with SSNS (vs SRNS) were enriched in genes pertaining to the cell cycle and the targets of microRNAs MIR106B and MIR16, in addition to those related to cytokines. The emergence of cell cycle-related genes may imply differences in the proliferative properties of SSNS and SRNS, which could be utilized for the development of novel therapeutic options. The 23 genes that were selected as markers of steroid responsiveness seem heterogeneous, but following refining with different sets of samples for validation, list of genes or part of this list can be used as markers of steroid responsiveness. Interestingly, comparison of the signature genes of SSNS with those listed as SSNS in the patent for “Kit and method for identifying individual responsiveness to steroid therapy of nephrotic syndrome”\(^{41}\) did not reveal any common genes, despite the similarity of the methods, indicating that clinical utilization of this approach requires further study. Notably, previously proposed circulating factors indicative of SRNS (cardiotrophin-like cytokine factor 1\(^{18}\) and urokinase-type plasminogen activator

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**Table 4.** Enriched Gene Ontology (GO) terms from K-means clustering of differentially expressed genes (DEGs) in steroid sensitive paediatric idiopathic nephrotic syndrome (SSNS). Clusters listed based on hypergeometric test of \(P < 0.005\).  

| Cluster | Genes | Enriched GO term(s) | Statistical significance |
|---------|-------|---------------------|-------------------------|
| Cluster 1, \(n = 6\) | BMPR1B, ITGA4, LOC255411, ODF2, PLA2G5, TCF4 | SMAD binding, Phospholipase A2 activity | \(P = 0.0039\) \(P = 0.0042\) \(P = 0.0007\) \(P = 0.0036\) |
| Cluster 3, \(n = 3\) | MSRB3, ZNF556, ZXDC | Oxidoreductase activity acting on sulphur group of donors | |
| Cluster 5, \(n = 6\) | ATCAY, LGALS4, PDE, RAD98, SAP300P, ZNF251 | N-terminal protein amino acid modification | |
receptor\textsuperscript{17}) were not found among the DEGs in the present study, possibly due to the heterogeneous nature of our study population. These proposed circulating factors were discovered in patients with recurrent NS after kidney transplantation in which steroid treatment can achieve remission in the majority of patients.

The present study has several shortcomings. First, the sample size was small, limiting the statistical power. Additionally, some relevant DEGs may not have been identified due to this small sample size. The DEGs identified in this study were able to clearly classify the groups, so our approach seems valid and justifies further studies to identify disease/therapeutic response markers for clinical applications. Secondly, although RNA sequencing was used rather than mRNA microarrays, DEIs and alternative splicing pattern differences between groups were not identified. To discover novel splice sites and rare transcripts, deep sequencing of at least 100 million reads of 76 bp in length is required (according to the guidelines of the Encyclopaedia of DNA Elements Project\textsuperscript{42}). The insufficient number of reads of this study (mean 77 million reads with up to 75% of the reads properly aligned against a reference genome) could be the reason for the failure in the DEI search, in addition to the small number of samples per group. Finally, the validation of candidate markers of NS or steroid responsiveness was not performed in this study. Clearly, many of the DEGs are not linked to pathogenesis but rather are the results of or surrogate changes due to disease. A validation study may be helpful in discriminating these differences.

In conclusion, whole-transcriptome sequencing of PBMCs found that DEGs of NS were enriched in immune system signaling, and potential therapeutic targets were suggested. Further studies with larger numbers of patients will provide more comprehensive information to enable the application of precision medicine to paediatric NS.

Declaration of Conflicting Interests

The authors declare that there are no conflicts of interest.

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