

# Abnormal RNA Processing and Altered Expression of Serin-Rich Proteins in Minimal-Change Nephrotic Syndrome

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## ABSTRACT

Mechanisms underlying the pathophysiology of minimal-change nephrotic syndrome (MCNS), the most frequent glomerular disease in children, remain elusive, but recent findings argue for a T cell dysfunction. Starting from a differential cDNA library from T cells of a patient under relapse and remission, we identified 16 transcripts specific for MCNS. All of these transcripts that were selectively up-regulated during the relapse phase of the disease were generated by alternative splicing of known genes. This abnormal RNA expression was associated with a down-regulation of serin-rich protein 75 and serin-rich

protein 40, two proteins involved in mRNA splicing. Taken together, these data suggest that T cell dysfunction in MCNS is associated with abnormal mRNA splicing. (*Pediatr Res* 57: 133–137, 2005)

### Abbreviations

MCNS, minimal-change nephrotic syndrome  
PBMC, peripheral blood mononuclear cell  
SR, serin-rich

Our understanding of biologic mechanisms that govern physiologic functions as well as pathologic situations has become more complex with recent insights from genome sequencing projects. In particular, it seems that alternative splicing plays a determinant role in the diversity and the complexity of higher organisms (1).

Alternative splicing displays a strong tissue specificity or involvement at a defined developmental stage (2). Recent genome-wide data of human alternative pre-mRNA splicing using exon-junction microarrays indicate that at least 74% of human multi-exon genes are alternatively spliced (3). Moreover, that alternative splicing plays a crucial role in the biologic complexity of vertebrates indicates the possibility of its involvement in a large number of human diseases or pathologic processes. Thus, it has been estimated that 15–20% of disease-causing mutations in human genes involve misregulation of alternative splicing (4).

In this report, we reveal the possible involvement of abnormal RNA processing in the pathogenesis of minimal-change

nephrotic syndrome (MCNS). This human glomerular disease is characterized by a heavy proteinuria with a relapsing/remitting course without histologic evidence of classical immune mechanism-mediated injury (5,6). MCNS is a sporadic disease that occurs in the context of immune activation, and its natural course argues strongly against a fixed genetic defect. It is believed that the mechanism of proteinuria involves the secretion by MCNS immune cells of factors that impair the glomerular filtration barrier (7). How the immune response culminates in MCNS by the production of pathogenic factor remains unresolved.

Recently, by analyzing a differential T cell cDNA library of a patient under relapse and remission, we identified 16 clones corresponding to unknown transcripts. The nucleotide sequences of these clones all match within intronic sequences of genes with defined or putative functions. Finally, we showed that this abnormal RNA processing is associated with a down-regulation of serin-rich protein 75 (SRp75) and SRp40 proteins that play a crucial role in pre-mRNA processing.

## METHODS

**Patients.** The cohort of patients analyzed in this study has been described previously (8). In all cases, informed consent was obtained, and the study was approved by the applicable Institutional Review Board.

**Cell isolation, immunocytochemistry, and reagents.** Immunoselection of T cell subsets and antibody reagents as well as preparation and quantification of protein

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extracts and SDS-PAGE immunoblotting were performed as previously described (6). Anti-SR protein Mab and Mab 104 were obtained from ATCC.

**RNA isolation, RT-PCR, and DNA sequencing.** Total RNAs were treated by DNase I and purified using RNeasy kit (Qiagen SA Courtabouef, France), following the supplier's protocol. Semiquantitative RT-PCRs and DNA sequencing were performed as previously reported (8). The sequence of primers and PCR parameters are indicated in Table 1. PCR reactions were normalized for glyceraldehyde-3-phosphate dehydrogenase expression. Nucleic acid and protein database searches were performed using resources of the National Center Biotechnology Information and the UCSC Genome Bioinformatics site ([www.genome.ucsc.edu](http://www.genome.ucsc.edu))

**Library construction and screening.** Total RNA from T cell-enriched peripheral blood mononuclear cells (PBMCs) were purified and DNase I-treated as previously described (8). First-strand cDNA synthesis was performed with Superscript reverse transcriptase (200 UI) using 4  $\mu$ g of total RNA and 2  $\mu$ M of 5'-AAGCAGTGGTATCAACGCAGAGTGGC-CATTATGGCCGGG-3' and 3'-ATTCTAGAGGCCGAGGCCGCCG-CATG-dT(30)-5' oligonucleotides. After RNA lysis, double-strand cDNA synthesis was performed by primer extension (30 min, 72°C) in 50  $\mu$ L including the total first-strand reaction product, 0.2  $\mu$ M of 5'-AAGCAGTGGTATCAACGCAGAGT and 5'-ATTCTAGAGGCCGAGGCCGCCG-CATG-3' PCR primers, 2 mM dNTPs, and 2  $\mu$ L of *Taq*DNA polymerase.

**Table 1.** Sets of primers used in semiquantitative RT-PCR

Clone number	GenBank accession number	Primers	Expected size	Annealing temperature	PCR cycles
c11	AY172689	S: 5'-CCTGCGAGGAGGGAAGTTACAGATCTC-3' AS: 5'-CAGGTAGCTATTGGCAGCCTGCAGTAAG-3' I: 5'-ATGGGACAGGCTGCTGAGCCAACT-3'	209	62	32
c12	BU744278	S: 5'-GTGAGACTTTAATTGCCAAGTTAACAAGCC-3' AS: 5'-TGATTGGTTTCCATACAGTGAAGTATTTC-3' I: 5'-CTCTTTAACATTTATGAACTTAGGTGT-3'	250	61	32
c13	BU744289	S: 5'-GACTAGGGTGATTTTCATCAGCATTGAG-3' AS: 5'-GGTTTGTACGACTGGACCTTATTAAGC-3' I: 5'-TCACCAACAAAATGAAGGTTTCTCCA-3'	110	58	32
c14	BU744279	S: 5'-TGAGCCTTTAACTGAACACAGATACAGG-3' AS: 5'-ATTCAGGGTGTGGTTTGGATGCACCTAC-3' I: 5'-ATATCGGAAGTTCCTATATTTTCATGC-3'	198	59	32
c15	BU744282	S: 5'-GGCAAGACTACTTCTTGATAGTG-3' AS: 5'-ACTGTTGGAGGATTCTAGGAAGCC-3' I: 5'-GGAATATTATTCTAATAAGAACAACTCATCTA-3'	330	56	32
c16	BU744280	S: 5'-CATCTTATATAAAAGGGGGCCAAATCT-3' AS: 5'-TGGACCAACCCATAGGAAGCCATAGTTC5-3' I: 5'-CAGGAAATTCACCTGGGACGTAATCCCT-3'	200	60	32
c17	BU744281	S: 5'TATACTATATACATCTTAATTGCCACAAACATTTCT-3' AS: 5'-CACTCCGACAGTAAATGCCAATAAAGGTG-3' I: 5'-TAAGGTTTTTAAAAAATTCTATTTTGCTTCT-3'	280	63	34
c18	BU744283	S: 5'-TATCGATGGCAATCTTGGATAGAGGC-3' AS: 5'-TGACCTACCCTTTAAACAACACACG-3' I: 5'-AAGTGGTGTTAATGACCTACCTGCACCGA-3'	290	61	32
c19	BU744288	S: 5'-CTACTAAGTGTCTATTGGGAGAATGAG-3' AS: 5'-CCGCGTACAAAATTAACAATTACA-3' I: 5'-ATTTTTAATCTGATTTTCACTATCT-3'	100	53	36
c110	BU744292	S: 5'-CACAGACCTAGGGAGTAAGAGTTGGGAA-3' AS: 5'-TCTCTGCCATTCATTAACATTTGTTAGATA-3' I: 5'-GTTTTTCACATCTATATTGTTAAAGG-3'	230	59	32
c111	BU744284	S: 5'-GGCAAGACTACTTCTTGATAGTG-3' AS: 5'-ACTGTTGGAGGATTCTAGGAAGCC-3' I: 5'-GGAATATTATTCTAATAAGAACAACTCATCTA-3'	330	56	32
c112	BU744290	S: 5'-GTATGAACATGAGGGTGTCTTCTCGTGTGAT-3' AS: 5'-GGAGAAGTCTCTGTGCTAGTAACCACGTTTC-3' I: 5'-CATTGTGTTGTGGTAAATATGTAGAGGGA-3'	200	60	32
c113	BU744291	S: 5'-CCCGAAAATCCCGGATTATATACA-3' AS: 5'-GCTTCAGTATTACTTATTGAGGGCATTTCAG-3' I: 5'-TGAATTACTGGAGTGGGTAGTGAGTGTACTT-3'	280	60	32
c114	BU744287	S: 5'-GAGACCTCTCATGAGTTCAGCCTCAC-3' AS: 5'TAGAAAATTGTGATGAAGTAAGTATACAGTCCCTCA3' I: s'-CAAGGCCTTAAATTAACCAACATTCOAAGA-3'	280	61	32
c115	BU744285	S: 5'-GTATTAAGAAAATGCAAAGGGCCAAGAC-3' AS: 5'-CCATTTAACCTCTTTGTTCTTATGGG-3' I: 5'-ACTTCCTGTGTTATAGTTCTAAGCCTAAT-3'	250	59	32
c16	BU744286	S: 5'-TAGGTAACCAACACTGAAAACCTTTGTAGT-3' AS: 5'-TGCTATCAAGACACAGGATTTTTTCC-3' I: 5'-CTGTTAGCATAAAGTCGTTGGGGGGCA-3'	360	56	32
GAPDH	NM004048	S: 5'-ACCACAGTCCATGCCATCAC-3' AS: 5'-TCCACCACCTGTTGCTGTGA-3' I: 5'-CTCAAGGGCATCTGGGCTACACTGAGCAC-3'	374	60	23

Sense (S) and antisense (AS) oligonucleotides were selected from the cDNA sequences of unknown clones (cl), the accession numbers of which are indicated. The size of each amplified product and its annealing temperature and the number of PCR cycles are indicated (I, internal oligonucleotide probe).

Double-strand cDNA was purified on a chromaspin-100 column (Clontech, Heidelberg, Germany), then ligated with *EcoRI-Sall-NoI* adapters (Clontech). Molecules longer than 400 bp were selected by size fractionation on a chromaspin-400 and ligated into the *EcoRI* site of the Bluescript SK(+) plasmid (Stratagene, Amsterdam, The Netherlands). One fifth of the ligation reaction was used to transform 100  $\mu$ L of XL1 blue ultracompetent cells by heat shock (Stratagene). Selected subtracted inserts obtained previously were used for screening the library according to a protocol already described (8).

## RESULTS

**Specific recruitment of unknown transcripts in MCNS patients.** The cDNA subtractive library from MCNS T cells of patients under relapse and remission revealed 16 cDNA clones related to yet unknown transcripts. Before proceeding with any further studies, we wanted to check the specificity of these cDNA for MCNS patients. Thus, we analyzed by semiquantitative RT-PCR the expression level of these transcripts in 10 patients with MCNS, clinical characteristics of which have been previously described (9). We found an increased and specific expression of all of these transcripts in MCNS patients, whereas normal subjects or patients with membranous nephropathy (MN) exhibited very modest levels of these RNA forms (Fig. 1A). In all patients but one tested, the expression level of these transcripts was increased in relapse *versus* remission. To determine whether the expression of these transcripts was preferentially restricted to PBMC subset, we purified CD4 and non-CD4 T cell fractions by negative immunomagnetic selection from PBMCs of patients who were in relapse. The RT-PCR analyses of six transcripts (c11, c12, c14, c19, c110, and c111) were performed on these cell populations and revealed that the highest level of these cDNA was observed in the CD4<sup>+</sup> T cell subset. As an example, the expression of c12 and 9 are shown in Figure 1B. These results suggest

that MCNS relapse is associated with generation of abnormal RNA expression that preferentially occurs in CD4<sup>+</sup> T cells.

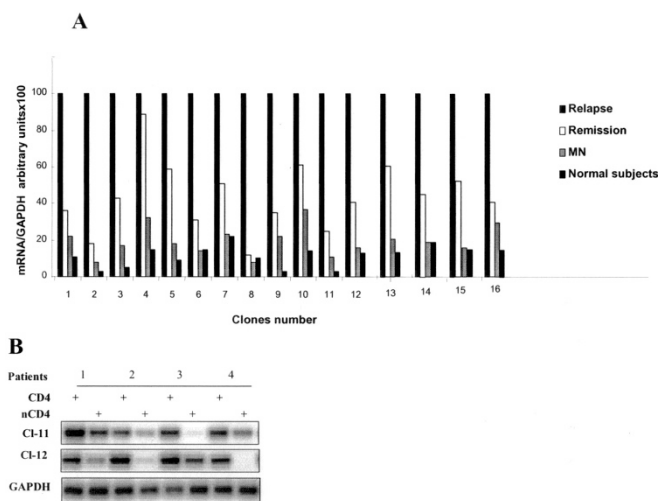
The alignment of these cDNA sequences with sequences available in Genbank revealed that all of these transcripts either matched with intronic regions or were spanning an intron within different genes. These genes are involved in various functions, including cell-cycle regulation and differentiation, cytoskeletal organization, transcription factors, and regulation of proteasome activity and cytokines (Table 2). To assess whether the presence of these modified transcripts leads to the production of altered protein in MCNS, we next analyzed by Western blot the expression patterns of the proteins encoded by the KIAA1694, ubiquitin-specific protease 16, actin-related protein type 2, and Runx1 genes that gave rise to c11, c12, c13, and c14 and detected an additional truncated form of the protein exclusively in MCNS patients (data not shown).

**Abnormal RNA expression was associated with down-regulation of SR proteins in MCNS relapse.** The modification of several transcripts in MCNS led us to investigate the expression of SR proteins. Pre-mRNA splicing takes place in the nucleus and involves SR proteins, a group of presently known nine proteins that contain domains rich in arginine and serine residues (RS domains). SR proteins are essential for constitutive and alternative splicing and have also been implicated in steps downstream of pre-mRNA splicing, including mRNA nuclear-cytoplasmic transport. We analyzed expression of SR proteins in normal resting and activated T cells as well as in 10 nonactivated T cell samples from MCNS patients, using the MAb 104, which recognizes primarily phosphorylated RS domains. We detected two major bands, each consisting of a doublet, corresponding to SRp75 and SRp40. Normal resting T cell expressed a low level of SR proteins. However, upon activation with anti-CD3/CD28 antibodies, the upper bands of the doublets, corresponding to phosphorylated forms, were increased, whereas the nonphosphorylated forms were barely detectable (Fig. 2A). That only SRp75 and SRp40 were detected after 36 h of stimulation is in accordance with previous studies showing that expression of other SR proteins occurs only after 72 h of stimulation. In all 10 patients who were in MCNS relapse, we were not able to detect any signal. Therefore, MCNS relapses were associated with down-regulation of SR proteins when compared with either activated or nonactivated normal T cells.

To further elucidate whether the lack of expression of SRp40 and SRp75 proteins was due to a defect of gene transcription, we analyzed the expression of SRp75 and SRp40 mRNA levels in T cells of MCNS patients by semiquantitative RT-PCR (Fig. 2B). Expression of SRp75 and SRp40 transcripts in MCNS patients and normal subjects was similar. Taken together, these results suggest that alteration of splicing RNA in MCNS was associated with translational inhibition of SRp75 and SRp40 transcripts.

## DISCUSSION

In the present study, we reported the preliminary identification and characterization of 16 transcripts with an altered expression in the T cells of MCNS patients. The specificity of



**Figure 1.** Selective induction of unknown transcripts in patients with MCNS relapse. (A) Semiquantitative RT-PCR analyses of 16 transcripts (c1–c16). PCR reactions were performed using primers specific for each cDNA clone as listed in Table 1. Quantification of PCR products was determined using the Image Quant V 1.11 analysis software. Values for each transcript are given as a bar graph after normalization against the amount of corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. (B) Expression of c10 and c11 in CD4<sup>+</sup> and non-CD4<sup>+</sup> (nCD4) subsets purified from PBMC as described in “Methods.” The expression of GAPDH was monitored in parallel.



other pathogen agents during the course of the disease. These studies suggest the presence of a soluble factor inhibiting T cell activation and proliferation (15–17).

Here we demonstrate that abnormal mRNA splicing occurs mainly in CD4<sup>+</sup> T cells from patients with MCNS. Whether the lack of expression of SR proteins in MCNS is related to the T cell anergic status remains an open question. Functional implications of the RNA processing anomalies during MCNS remain to be determined as well. We have previously demonstrated that alternative splicing of the KIAA1694 gene leads to a truncated protein named Tc-mip (truncated c-maf inducing protein) and is involved in the Th2 signaling pathway of CD4<sup>+</sup> T cells (9). The significance of other mRNAs' alternative splicing forms in the context of pathogenesis of MCNS remain to be determined.

Patients with MCNS tend to have high IgM and low IgG levels. Similar to alteration in SR protein expression during MCNS, these abnormalities are reversible and considered a defect in Ig switching (18). The immunoglobulin RNA-processing regulation is still poorly understood. However, it was demonstrated recently that pre-mRNA processing during B cell development is highly sensitive to artificial change in the SR protein expression (19). Whether defect in Ig class switching during MCNS relapse is related to alteration in SR protein remains unresolved but constitutes an exiting challenge in the future.

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