ACTA ABP BIOCHIMICA POLONICA

Vol. 63, No 1/2016
131–138
http://dx.doi.org/10.18388/abp.2015_1105

Regular paper

SOCS3 is epigenetically up-regulated in steroid resistant nephrotic children

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Background. **The mechanism of steroid resistance in children with the nephrotic syndrome is yet unknown. About 20% of patients demonstrate steroid unresponsiveness and progress to end stage renal disease. Aberrant *SOCS3* and *SOCS5* expression in steroid resistant and sensitive patients has previously been demonstrated. Here, we investigate genetic and epigenetic mechanisms of regulation of *SOCS3* and *SOCS5* transcription in nephrotic children. Methods. 76 patients with the nephrotic syndrome (40 steroid resistant and 36 steroid sensitive) and 33 matched controls were included in this study. We performed genotyping of a total of 34 single nucleotide polymorphisms for SOCS3 and SOCS5 promoters and evaluated their methylation status using MS-PCR and QMSP methods. Results**. **Steroid resistant patients had a significantly lower methylation of one region of SOCS3 promoter in comparison with steroid sensitive patients and controls ($p < 0.0001$). However, the relative methylation level in the steroid sensitive patients and controls differed significantly even before the first steroid dose ($p = 0.001758$). Other SOCS3 and SOCS5 promoter regions displayed no differences in methylation or were fully methylated/unmethylated in all study groups, showing site-specific methylation. The allele and genotype distribution for *SOCS3* and *SOCS5* markers did not differ statistically between the groups. Conclusions. We demonstrate an epigenetic mechanism of *SOCS3* up-regulation in steroid resistant children with the nephrotic syndrome. The assessment of methylation/unmethylation of SOCS3 promoter might be an early marker for steroid responsiveness in NS patients.**

Key words: methylation, nephrotic syndrome, single nucleotide polymorphism, steroid resistance

Received: 09 July, 2015; revised: 18 November, 2015; accepted: 18 December, 2015; available on-line: 01 February, 2016

INTRODUCTION

Idiopathic nephrotic syndrome (INS) in children is characterized by massive proteinuria and generalized oedema (Bagga & Mantan, 2005). Its incidence is estimated at 2–7/100 000 annually and depends on age, gender and ethnicity (Holt &Webb, 2002). A wide spectrum of histopathological features has been described in biopsy specimens from nephrotic patients and the pathogenesis is most often attributed to abnormalities in the immune responses and aberrant cytokine signaling, including the Jak/Stat pathway defects, leading to chronic inflammation and renal fibrosis (Gómez-Guerro *et al.*, 2004; Huang *et al.*, 2008; Thomas, 2009). Management of nephrotic patients is based on oral glucocorticosteroids (GCs). Patients typically respond to the steroid treatment, although they often require prolonged or alternative therapy due to recurrent relapses, steroid dependence or side effects. Still, up to 20% of patients manifest steroid unresponsiveness and are at a particular risk of high doses of steroids and rapid progression to end stage renal disease (Holt & Webb, 2002; Bagga & Mantan, 2005). Numerous studies have been conducted to reveal the mechanism of steroid resistance and to determine its early biomarkers. Several factors, including gene mutations and polymorphisms, have been attributed to steroid unresponsiveness, however, the pathophysiology of INS and the mechanisms of steroid resistance still remain to be elucidated (Hinkes *et al.*, 2008, Jafar *et al.*, 2011).

Our previous study demonstrated significant up-regulation of *SOCS3* and *SOCS5* in steroid resistant nephrotic patients when compared to steroid sensitive patients and normal controls (Ostalska-Nowicka *et al.*, 2011). Numerous diseases have been attributed to immunological perturbations and abnormal SOCS inhibitors activity, particularly their anti-inflammatory and tumor suppressor functions have been implicated in a variety of inflammatory conditions and cancers (Delcuve *et al.*, 2009; Zhang *et al.*, 2009). Gene expression and mechanisms of promoter regulation have been studied extensively. Recently, it has been reported that gene promoters may be actively methylated and demethylated (Metivier *et al.*, 2008). Although the epigenetic mechanisms seem to be of the greatest importance for the regulation of gene transcription, genetic alterations in the structure of gene body are also significant (Stenvinkel *et al.*, 2007). Special attention has been paid to genetic and epigenetic studies in the scope of biomarkers of drug response and personalized treatment, also in nephrology.

Therefore, the aim of this study was to examine genetic and epigenetic mechanisms of previously report-

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Abbreviations: AP-1, Activator protein-1; bp, base pair; Dnmt, DNA methyltransferase; EDTA, Ethylenediaminetetraacetic acid; F, Forward; GCs, Glucocorticosteroids; IFN-gamma, Interferon-gamma; INS, Idiopathic Nephrotic Syndrome; ISKDC, International Study of Kidney Disease in Children; MAF, Minor Allele Frequency; MSP, Methylation Specific PCR; NS, Nephrotic Syndrome; nt, nucleotide; PBMC, Peripheral Blood Mononuclear Cell; PCR, Polymerase Chain Reaction; QMSP, Quantitative Methylation-Specific PCR; R, Reverse; RA, Rheumatoid Arthritis; SNP, Single Nucleotide Polymorphism; SOCS, Suppressor of Cytokine Signaling; Sp1, Specificity protein 1; SR, Steroid Resistant; SS, Steroid Sensitive; STAT, Signal Transducer and Activator of Transcription; Th, T helper cell; UTR, Untranslated Region

ed *SOCS3* and *SOCS5* up-regulation in steroid resistant children with INS. We conducted SNP genotyping in promoters of *SOCS3* and *SOCS5* and estimated the methylation status of their CpG islands.

SUBJECTS AND METHODS

Study Subjects. We recruited 76 Caucasian children (39 males (51.3%) and 37 females (48.7%)) diagnosed with NS for our study. All patients were followed up at the Clinic of Cardiology and Nephrology, University of Medical Sciences in Poznan, Poznań, Poland. The age of patients ranged from 3 months to 16 years (mean 3.9 ±S.D. 3.4 years). All patients were treated according to standard ISKDC (International Study of Kidney Disease in Children) regimen for the first NS episode, as reported previously (Ostalska-Nowicka *et al.*, 2011). After administration of drugs, all patients were categorized into two subgroups, depending on the results of initial treatment: primary steroid resistant (SR) subgroup (40 patients: 23 males (57.5%) and 17 females (42.5%)) and primary steroid sensitive (SS) subgroup (36 patients: 16 males (44.4%) and 20 females (55.6%)). Steroid sensitivity and resistance were defined according to the ISKDC standards (Banaszak & Banaszak, 2012).

33 healthy children (20 males (60.6%) and 13 females (39.4%)) without renal diseases were recruited as the control group and were sex, age and ethnically matched.

Analysis protocol was approved by the Ethics Committee of Poznan University of Medical Sciences and the recruitment of patients was performed after their parents gave signed consents.

SNPs genotyping. Genomic DNA was extracted from the whole blood, collected in EDTA vials during hospitalization of patients, before the first steroid dose. DNA extraction was performed using Quick Blood DNA Purification Kit (EURx), according to the manufacturer's protocol.

To determine the functional promoter regions of SOCS3 (GeneBank:9021) and SOCS5 (GeneBank:9655) for our analysis we used Cister (Cis-element cluster finder) software (http://zlab.bu.edu/~mfrith/cister.shtml) and the literature review (Paul *et al.*, 2000; He *et al.*, 2003a; Ehlting *et al.*, 2005). 6 single nucleotide polymorphisms for SOCS3 and 27 SNPs for SOCS5 were genotyped. All SNPs were assessed by the Ensembl database (http://www.ensembl.org) and are listed in Table 1. We performed Sanger sequencing using primers designed with Primer 3. Table 2 shows primers used in PCR analysis for amplification of

Table 1. Single nucleotide polymorphisms for SOCS3 and SOCS5 analyzed in this study. MAF, minor allele frequency; *when available

	Name of variation	MAF *	Location	Base pair position	nt change
SOCS3					
1	rs111033850	0.06	5′UTR	Chr17:78360132	T>C
2	rs12953258	0.18	exon 1 (UTR)	Chr17:78360015	C>A
3	rs192803725	0.01	exon 1 (UTR)	Chr17:78359894	C>A
4	rs116303707	<0.01	intron 1	Chr17:78359604	C>G
5	rs148699063	<0.01	intron 1	Chr17:78359431	C>A
6	rs113849007		intron 1	Chr17:78359325	C>G
SOCS5					
1	rs41452946	0.01	5′UTR	Chr2:46698240	C>T
2	rs35750425	0.07	5′UTR	Chr2:46698373	G>T
3	rs13386416		5′UTR	Chr2:46698433	A>T
4	rs190344190	<0.01	5′UTR	Chr2:46698486	C>A
5	rs376147440		5′UTR	Chr2:46698531	G>C
6	rs41501846	0.01	5′UTR	Chr2:46698533	T>C
7	rs4952842	0.22	5′UTR	Chr2:46698591	C>A
8	rs185459620	<0.01	5′UTR	Chr2:46698608	G>A
9	rs41389052	0.07	5′UTR	Chr2:46698642	A>C
10	rs3829835	0.23	5′UTR	Chr2:46698650	C>T
11	rs189872419	<0.01	5′UTR	Chr2:46698671	T>C
12	rs3814040		5′UTR	Chr2:46698720	C>T
13	rs41504048	0.01	5′UTR	Chr2:46698721	C>A
14	rs77033967		5′UTR	Chr2:46698726	C>T
15	rs41417248		5′UTR	Chr2:46698895	G>A
16	rs3814039	0.29	5′UTR	Chr2:46698925	C>G
17	rs13000826		exon 1 (UTR)	Chr2:46699007	C>G
18	rs111677684		intron 1	Chr2:46699283	T>C
19	rs79753377	<0.01	intron 1	Chr2:46758098	T>C
20	rs41320649		intron 1	Chr2:46758106	T>A
21	rs34642457		intron 1	Chr2: 46758168 - 46758169	- >C
22	rs200381087		intron 1	Chr2:46758338	C>G
23	rs41428947	0.01	intron 1	Chr2:46758353	T>C
24	rs41483445	0.04	intron 1	Chr2:46758405	G>A
25	rs148852176	<0.01	intron 1	Chr2:46758451	C>G
26	rs369277165		intron 1	Chr2:46758512	T>G
27	rs372918921		exon 2 (UTR)	Chr2:46758527	A>G

Table2. Primer sequences for analysis of *SOCS3* and *SOCS5* polymorphisms.
F, Forward; R, Reverse. Amplified region estimated as nucleotide number in reference to ATG triplet.

Region	Sequences	Annealing temp./elongation
SOCS3		
–1397/–1054	F: 5′-CAGGTCGGCCTCCTAGAACT-3′	59°C/30 s
	R: 5′-CCGGCCTTCTTGTAATGTTT-3′	
–1201/–855	F: 5′-CTCTCGTCGCGCTTTGTCT-3′	59°C/30 s
	R: 5′-GGGAGGGGACCAGGAGAG-3′	
–977/–428	F: 5′-CGACTTGGACTCCCTGCTC-3′	61°C/60 s
	R: 5′-GTGTGGACGGAGGGAGAAAC-3′	
–521/+23	F: 5′-ATCCCAGGTTCCCGGAATAC-3′	60°C/60 s
	R: 5′-GGAAACTTGCTGTGGGTGAC-3′	
–361/+23	F: 5′-GCCACACTCCTGGAGACCTA-3′	60°C/60 s
	F: 5′-GGAAACTTGCTGTGGGTGAC-3′	
SOCS5		
–60351/–59851	F: 5′-GGTACGTTTGTGAACGACGA-3′	60°C/60 s
	R: 5′-GAGAGGAAAGTGCTGAATGGA-3′	
–60042/–59481	F: 5′-CCCTCCGATTTGTGAGTCAT-3′	60°C/60 s
	R: 5′-GTAGGTGAAGGCCGAAGGAG-3′	
–59598/–59150	F: 5′-AGCTGCCAGACTCCAAAATG-3′	59°C/60 s
	R: 5′-GTCACCGACAGGGCGAGT-3′	
–471/+81	F: 5′-GGGAAGATTGCTACTAATGA-AAGG-3′	60°C/60 s
	R: 5′-ACTACGGCTTCCTCCCTCAT-3′	

overlapping regions. The reaction mastermix contained 30–60 ng of DNA, 200 μM of dNTPs, 0.5 μM of each primer, 1x PCR buffer and 1U of FastStart DNA polymerase (Roche) in a final volume of 12.5 μl. The reactions were performed in Veriti 96 Well Thermal Cycler (Applied Biosystems) and the conditions were: 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, specific annealing temperature for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were purified using 96-well membrane plates (Millipore) and used as templates in sequencing-PCR. The latter reaction was performed with only one primer (Forward or Reverse) and products were purified using EDTA and ethanol precipitation, according to the Life Technologies protocol. Formamide (Applied Biosystems) was used for denaturation of samples and then they were separated by capillary electrophsoresis using ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

Methylation status of SOCS3 and SOCS5 CpG islands. 1 μg of genomic DNA was used for bisulfite conversion using commercially available EZ DNA Methylation Kit (Zymo Research). Methylation status of *SOCS3* and *SOCS5* CpG islands was investigated by methylation-specific polymerase chain reaction (MSP-PCR) as previously reported (Wojdacz & Dobrovic, 2007). We used Methyl Primer Express v1.0 software to design MSP primers that specifically recognized the methylated and unmethylated sequence. A graphical overview of *SOCS3* and *SOCS5* is shown in Fig. 1. As previously described (Niwa *et al*., 2005; Fernández-Mercado *et al*., 2008; Zhang *et al*., 2013), methylation status may differ in the same sample depending on the selection of CpG island region, that is why we selected 3 representative regions for *SOCS3* and 1 region for *SOCS5*. MSP primers that recognized methylated and unmethylated sequences within *SOCS3* and *SOCS5* CpG islands are listed in Table 3. SOCS3.2 primers were adopted from Ghattas *et al*. (2013). The reaction was performed in a final volume of 12.5 μl, containing 1 μl of bisulfite-treated DNA, 200 μM of dNTPs, 0.5 μM of each primer, 1x PCR buffer and 1U of FastStart DNA polymerase (Roche). All amplifications were performed in Veriti 96 Well Thermal Cycler (Applied Biosystems) under conditions: initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, specific annealing temperature for 30 s, 72°C for 1 min, and a final extension at 72°C for 7 min 10 μl of MSP-PCR product was loaded on 2.5% agarose gel with ethidium bromide used for visualization after electrophoresis. We used Jurkat Genomic DNA and CpG Methylated Jurkat Genomic DNA (Thermo Scientific) as positive controls for unmethylated and methylated DNA, respectively.

Additionally, we performed quantitative methylation-specific PCR (QMSP) to determine the relative methylation level. The higher the number of CpG sites within the amplicon, the higher the Ct values and the difference of melting temperature between highly methylated and unmethylated DNA template (Wojdacz & Dobrovic, 2007). Here, we analyzed SOCS3.1 and SOCS3.2 regions for evaluation of Ct, as differences in the methylation status were shown for these regions (see: Results). QMSP was carried out using primers specific both for methylated and unmethylated sequences. The reaction was performed in a final volume of 20 μl, containing 4 μl of 5x Hot FirePol EvaGreen HRM Mix (Solis BioDyne), 0.25 μM of each primer and 1 μl of bisulfite-treated DNA. The amplification was carried out in 7900HT Fast Real-Time PCR System (Applied Biosystems) under conditions: initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 20 s.

Statistical analyses. Sequencing Analysis v5.2 was used to collect genotyping data. Genotype and allele frequencies for analyzed SNPs in *SOCS3* and *SOCS5* were tested by the chi-square test for deviation from Hardy-Weinberg and to compare gender variable, while the Fisher's exact test was used to investigate differences in genotype and allele frequencies between study groups. Comparisons between SR and SS patients and between NS patients (defined as SS and SR patients altogether) and controls were performed. *P*-values ≤ 0.05 were considered statistically significant.

Table 3. MS-PCR primer sequences for analysis of SOCS3 and SOCS5 CpG islands. M, methylated; U, Unmethylated; F, Forward; R, Reverse. Amplified region estimated as nucleotide number in reference to ATG triplet.

CpG region	Sequences	Product size
SOCS3.1	MF: 5′-ATTATAAGAAGGTCGGTCGC-3′	139 bp
–1070/–926	MR: 5′-CTAACTACGTACGAAACCGAA-3′	
	UF: 5′-AATATTATAAGAAGGTTGGTTGT-3′	145 bp
	UR: 5′-ACTAACTACATACAAAACCAAAAC-3′	
SOCS3.2	MF: 5′-GGAGATTTTAGGTTTTCGGAATATTTC-3′	142 bp
–526/–385	MR: 5′-CCCCCGAAACTACCTAAACGCCG-3′	
	UF: 5′-GTTGGAGATTTTAGGTTTTTGGAATATTTT-3′	151 bp
	UR: 5′-AAACCCCCAAAACTACCTAAACACCA-3′	
SOCS3.3	MF: 5′-TTTTTGATTCGCGATAGTTC-3′	143 bp
202/344	MR: 5′-AACACGAACTACGTACTCCG-3′	
	UF: 5′-ATTTTTTTGATTTGTGATAGTTT-3′	146 bp
	UR: 5′-AACACAAACTACATACTCCAAAA-3′	
SOCS5	MF: 5′-ATGGTAGTTCGTAGAGCGC-3′	112 bp
–59566/–59460	MR: 5′-CGAAATTCCTAACGACCAAT-3′	
	UF: 5′-AAAATGGTAGTTTGTAGAGTGT-3′	118 bp
	UR: 5′-ACAAAATTCCTAACAACCAATCC-3′	

We used Fisher's exact test to evaluate the differences in methylation status between the study groups and *P*-values ≤ 0.05 were considered statistically significant. The Wilcoxon test was used to assess the differences in methylation status between the groups. All statistical analyses were performed in the R language.

RESULTS

SOCS3 and SOCS5 single nucleotide polymorphisms and LD analysis

We screened 76 NS patients and 33 controls for 6 SNPs in *SOCS3* and 27 SNPs in *SOCS5*. All genotypes were in Hardy-Weinberg equilibrium. Figure 1 shows graphical overview of genotyped polymorphisms. Out of all 33 SNPs submitted in this study, 26 were homozygous in all subjects and they were excluded from further statistical analyses. For 7 heterozygous SNPs (rs12953258 in *SOCS3* and rs35750425, rs4952842, rs41389052, rs3829835, rs3814039, rs41483445 in *SOCS5*) genotype/ allele frequencies were calculated and compared. Genotype and allele frequencies and p-values for heterozygous markers analyzed in the study are listed in supplementary Table 4 (at www.actabp.pl). There were no significant differences between study groups and between males and females in each group.

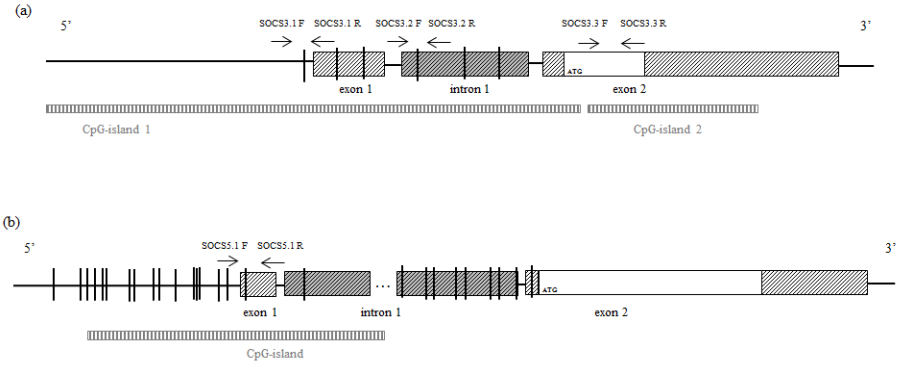

Figure 1. Schematic diagram of SOCS3 and SOCS5 genes analyzed in this study. Cross-hatched white rectangles represent untranslated regions, cross-hatched grey rectangles — introns and white rectangles — translated regions of exons. The translation start at the ATG triplet is marked. Vertical lines indicate polymorphisms analyzed in this study. CpG island extension is indicated as tripped grey rectangles below the diagrams. Gene regions amplified for methylation analysis are indicated by arrows, representing forward and reverse primers. (**a**) SOCS3; (**b**) SOCS5.

Methylation status of SOCS3 and SOCS5 promoters

MSP-PCR. The methylation-specific PCR results are shown in Table 5. All study subjects displayed positive methylation for SOCS3.1 promoter region. In the NS group, 5.3% (n = 4) showed full unmethylation (positive reaction only with the unmethylated primers), while 94.7% (n = 72) showed partial methylation (positive for both methylated and unmethylated primers). In the SR group, 5% (n = 2) of the samples were fully unmethylated and 95% (n = 38) showed partial methylation. In the SS group, 5.6% (n = 2) of the samples were fully unmethylated and 94.4% (n = 34) showed partial methylation. 3% (n = 1) of the samples in the controls were unmethylated, while 97% (n = 32) showed partial methylation. The differences in methylation status of SOCS3.1 promoter region were not statistically significant between the study groups ($p = 0.6132$ for NS patients *vs.* controls; $p = 0.9138$ for SR *vs.* SS patients).

A positive methylation for SOCS3.2 promoter region was also detected in all study subjects. In the NS group, 51.3% (n = 39) was fully unmethylated and 48.7% (n = 37) showed partial methylation. However, there was a strong significant difference between NS subgroups ($p < 0.0001$). In the SR group, 82.5% (n = 33) of patients showed fully unmethylated region 2, while 17.7% (n = 7) showed partial methylation. On the contrary, in the SS group, only 16.7% (n = 6) was fully unmethylated, while 83.3% (n = 30) showed partial methylation. In controls, 6.1% (n = 2) of the samples were fully unmethylated and 93.9% (n = 31) were partially methylated. The methylation status of SOCS3.2 region was not significantly different between the controls and SS patients ($p = 0.1861$), however there was a significant difference between SR and SS group ($p < 0.0001$) and between NS patients and controls ($p = 0.0003$).

For SOCS3.3 promoter region 100% of the samples of all study groups showed full methylation, while for SOCS5 promoter region a fully unmethylated

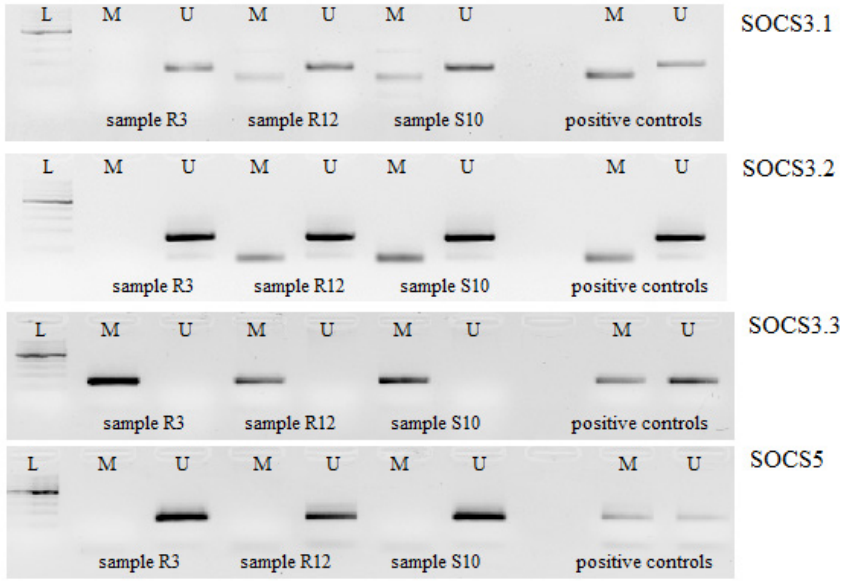

Figure 2. Representative data for gel-based methylation status of SOCS3 promoter (regions 1, 2 and 3) and of SOCS5 promoter (region 1).
M, methylated DNA; U, unmethylated DNA; L, 100–1000 bp DNA ladder; positive M and U controls (universal methylated and unmethylated DNA). Illustrative samples of two steroid resistant patients (R3 and R12) and one steroid sensitive patient (S10) are shown for visualization of methylation status of all promoter regions analyzed in this study. (**a**) region1 of SOCS3 promoter; (**b**) region2 of SOCS3 promoter; (**c**) region3 of SOCS3 promoter; (**d**) region1 of SOCS5 promoter.

(100%) pattern was observed. Figure 2 shows representative results of methylation status of SOCS3.1, SOCS3.2, SOCS3.3 and SOCS5.1 promoter regions. There were no differences in methylation pattern of any region of SOCS3 and SOCS5 promoters in reference to gender in the study groups (data not shown).

QMSP. Additionally, we analyzed a relative CpG methylation level calculated as the Ct value for SOCS3.1 and SOCS3.3 promoter regions. We performed Wilcoxon rank sum test with continuity correction as more efficient than the t-test for analysis of both regions (Fig. 3). There were no significant differences in methylation level of SOCS3.1 region between analyzed groups (Fig. 3c). Surprisingly, there was a significant difference between SR group and controls ($p = 1.013e\text{-}10$), as well as between SS group and controls ($p = 0.001758$), while no such observation was made for SR and SS comparison ($p = 0.8027$) (Fig. 3d).

Table 5. Gel-based methylation status of SOCS3 and SOCS5 promoter in NS patients and controls.
-/U, full unmethylation (positive reaction only with the unmethylated primers); M/U, partial methylation (positive for both methylated and unmethylated primers); M//-, full methylation (positive reaction only for methylated primers); The *P*-value represents fisher's exact test; ***statistically significant at $p \leq 0.001$.

Gene/Region		M/-	M/U	-/U	*P*-value	
SOCS3/						
SOCS3.1	NS n=76	0 (0%)	72 (94.7%)	4 (5.3%)	NS *vs.* C	0.6132
	SR n=40	0 (0%)	38 (95.0%)	2 (5.0%)	SR *vs.* SS	0.9138
	SS n=36	0 (0%)	34 (94.4%)	2 (5.6%)	SS *vs.* C	0.6126
	C n=33	0 (0%)	32 (97.0%)	1 (3.0%)	SR *vs.* C	0.6762
SOCS3.2	NS n=76	0 (0%)	37 (48.7%)	39 (51.3%)	NS *vs.* C	0.0003***
	SR n=40	0 (0%)	7 (17.5%)	33 (82.5%)	SR *vs.* SS	<0.0001***
	SS n=36	0 (0%)	30 (83.3%)	6 (16.7%)	SS *vs.* C	0.1861
	C n=33	0 (0%)	31 (93.9%)	2 (6.1%)	SR *vs.* C	<0.0001***
SOCS3.3	NS n=76	76 (100%)	0 (0%)	0 (0%)		
	SR n=40	40 (100%)	0 (0%)	0 (0%)		
	SS n=36	36 (100%)	0 (0%)	0 (0%)		
	C n=33	33 (100%)	0 (0%)	0 (0%)		
SOCS5/	NS n=76	0 (0%)	0 (0%)	76 (100%)		
SOCS5.1	SR n=40	0 (0%)	0 (0%)	40 (100%)		
	SS n=36	0 (0%)	0 (0%)	36 (100%)		
	C n=33	0 (0%)	0 (0%)	33 (100%)		

DISCUSSION

The scope of this study was to investigate the genetic and epigenetic status of the promoter regions of *SOCS3* and *SOCS5* genes and to correlate differences between genotype distribution and/or methylation status to steroid sensitivity or resistance in the course of NS in children. In a previous study (Ostalska-Nowicka *et al.*, 2011), we had demonstrated aberrant expression levels of *SOCS3* and *SOCS5* in monocytes and T cells,

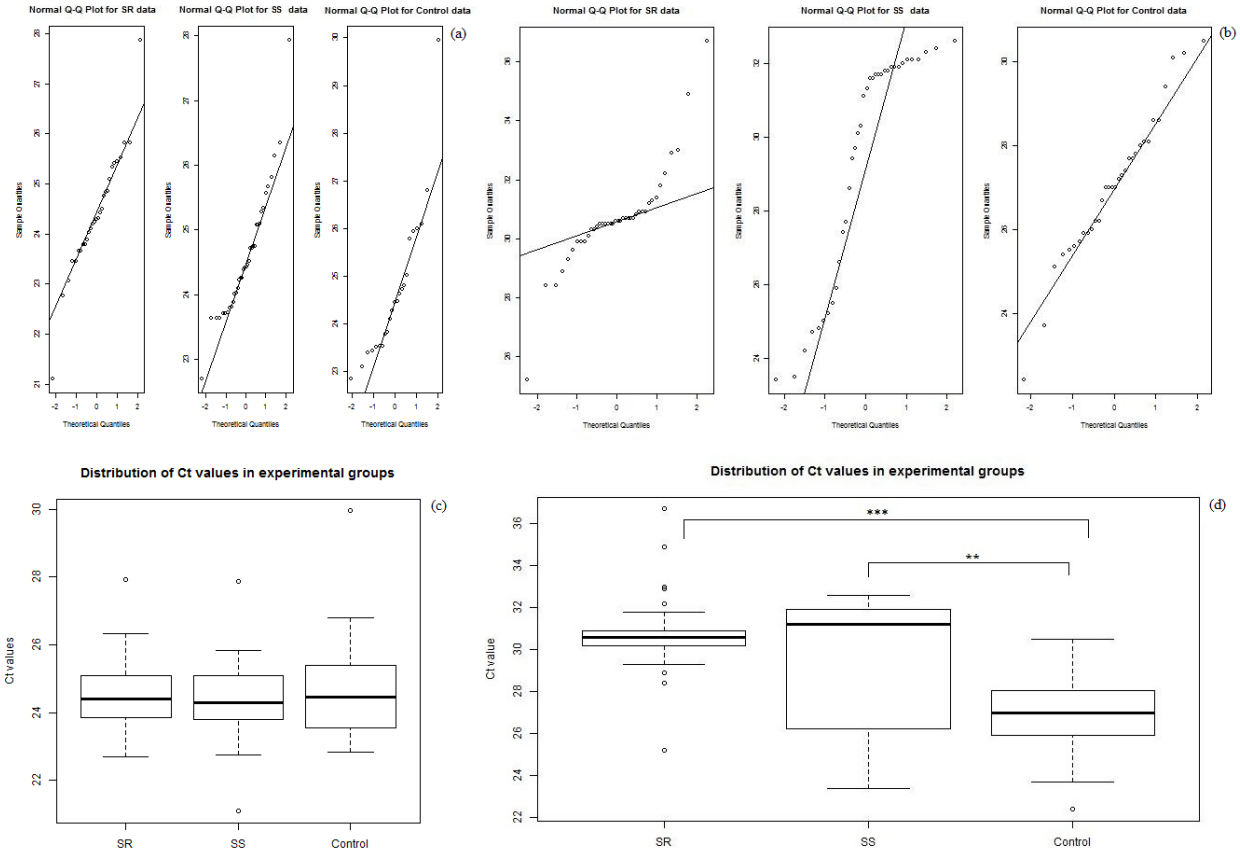

Figure 3. Relative methylation levels of SOCS3 promoter regions 1 and 2.
The results of the Wilcoxon ran sum test shown as Q-plots presenting non-normal distribution in SR, SS and control group for SOCS3.1 region (**a**) and for SOCS3.2 region (**b**). Distribution of Ct values shown as box-plots for SR, SS and control group for SOCS3.1 region (**c**) and for SOCS3.2 region (**d**). Significance at $p < 0.01$**, $p < 0.001$***.

respectively, in NS patients after administration of steroids. The analysis of single nucleotide polymorphisms in coding regions of both SOCS genes did not reveal any significant differences between steroid sensitive and resistant children (data not shown). Here we demonstrate that *SOCS3* expression might be epigenetically regulated in peripheral blood cells of NS patients due to a high level of demethylation of SOCS3 promoter region, which corresponded to *SOCS3* up-regulation in steroid resistant NS children.

The hypermethylation of promoter of suppressor genes, including SOCS factors, is a well known mechanism of gene silencing in many diseases (He *et al.*, 2003b; Stenvinkel *et al.*, 2007; Sobti *et al.*, 2011; Wilop *et al.*, 2011). Recently, more interest has been drawn to the role of promoter hypomethylation and its deregulative influence on genetic instability and promoting forced or prolonged gene expression in tumorigenesis and various immunological disturbances (Mi & Zeng, 2008). CpG methylation occurs to be an integral component of transcription and methylation-demethylation alteration is an active, rapid and cyclic process (Metivier *et al.*, 2008; Kobayashi *et al.*, 2012). Moreover, epigenetic patterns could not only distinguish between types of malignancies serving as specific markers, but could also alter drug sensitivity and affect drug resistance (Teodoridis *et al.*, 2004; Wojdacz & Dobrovic, 2007; Fernández-Mercado *et al.*, 2008; Ghattas, 2013).

It is still argued whether steroid resistance during the course of NS is an acquired mechanism resulting from rapidly changing inner immunity or a primary disease that results in increased cytokine signaling restricted to the kidney area, despite administration of anti-inflammatory drugs (Kam *et al.*, 1993). Circulating cytokines themselves have been shown to induce steroid resistance, observed in lymphocytes and monocytes (Barnes, 1998; Bantel *et al.*, 2002; Camici, 2007). Others (Stenvinkel *et al.*, 2007; Delcuve, 2009; Hodge *et al.*, 2011) have demonstrated that pro-inflammatory cytokines and chronic inflammation could influence DNA methylation mainly through the increased DNA methyltransferase (Dnmt) expression and activity. Ghattas *et al.* (Ghattas *et al.*, 2013) indicated an altered methylation pattern of IFN-gamma, SOCS1 and SOCS3 promoters in patients with chronic kidney disease in comparison with healthy controls. Gene expression levels also correlated with a severity of inflammation and progression to end stage renal disease. In this study, SOCS3 promoter was significantly hypomethylated in SR patients in comparison with SS patients and healthy controls. Interestingly, in our previous study SOCS3 was up-regulated in all NS patients before the first dose of steroids and only in steroid sensitive patients it was down-regulated to the level compared with controls, after administration of the drugs (Ostalska-Nowicka *et al.*, 2011). It is known that GCs exert their anti-inflammatory action mainly

through the negative regulation of T helper type 1 cells and modification of the immune response (Ramírez *et al.*, 1996; Berkley *et al.*, 2013). It is also well documented that active methylation/demethylation processes regulate activation and repression of genes responsible for CD4+ T cell differentiation and maintenance (Teitell & Richardson, 2003; Wilson *et al.*, 2005; Kino, 2007; Komatsuda *et al.*, 2008; Chaoran, 2014). Richardson (2003) showed that defective DNA methylation of Th cells resulted in diverse immune reactivity *in vitro* and *in vivo* in mice, resembling that of Th_2- and Th_1-type autoimmune diseases. Discordant methylation can therefore result in an excessive Th_1 or Th_2 polarization in SR and SS patients (respectively) during the course of NS, as well as in constant changes of immune response during remissions and relapses (Komatsuda *et al.*, 2008). Also, T cells from patients with rheumatoid arthritis have been reported to have hypomethylated DNA, but in contrast to lupus patients, T cells from RA patients demonstrated normal *Dnmt1* expression levels (Richardson, 2003). Dnmt expression analysis was beyond the scope of this study, however, its role in steroid resistance in NS needs further research.

Out of three analyzed SOCS3 promoter regions, only one (SOCS3.2) was significantly aberrantly methylated between SR and SS patients in this study (Table 4). Interestingly, the MSP method showed no statistical differences in methylation status of SOCS3.2 region between SS and control subjects, while using QMSP we were able to distinguish methylation levels between those groups. However, MSP indicates an overall pattern of methylation characterized by the presence or absence of any methylated CpG sites within a sequence, while the QMSP method is a relative quantification of promoter methylation, dependent on precise number of CpGs altering specific Ct value. Moreover, the relative methylation levels for SS and SR patients were comparable ($p = 0.8027$). However, it should be emphasized that the high Ct values in SR group most probably resulted from artifacts, as 82.5% of SR patients exhibited positive reaction only for unmethylated sequence (Table 5). Therefore, the high Ct values in SS group indicated a relatively high methylation level, as 83.3% of SS patients showed methylation-positive pattern in MSP (Table 5). The level of methylated CpG sites in SS patients was statistically higher than in the healthy controls, although there were no differences in *SOCS3* expression levels (Ostalska-Nowicka *et al.*, 2011). It may not be excluded that different methylation levels of SOCS3.2 region in SR and SS groups may be due to a distinct mechanism, which implies the steroid sensitive and steroid resistant nephrotic syndrome to be independent diseases, demonstrating different mechanisms underlying the inflammatory process and action of GCs. It seems likely that the primary and acquired steroid resistance could also result from different immune cell activities. Unfortunately, we were not able to examine DNA methylation status in NS patients after the GC treatment. Nevertheless, distinct methylation pattern for SOCS3.2 promoter region between steroid sensitive and resistant NS patients strongly suggests different mechanisms of regulation of *SOCS3* expression in both groups and should be taken under consideration when deciding on proper patient treatment.

When evaluating the methylation status, it is recommended to select primers that amplify a particular region of interest (Niwa *et al.*, 2005; Fernández-Mercado *et al.*, 2008; Zhang *et al.*, 2013). Surprisingly, in our study, no transcription factors known for SOCS3 appear to bind to promoter region SOCS3.2, while several, including AP-1, STAT1, STAT3 and Sp1 have been shown to bind to region SOCS3.1, which showed no significant differences in methylation status between the groups. Apart from promoter methylation, point mutations and polymorphisms also work together to regulate gene transcription. Single nucleotide change might create a novel CpG site or influence binding of transcription factors (Gluckman *et al.*, 2009). Several studies have demonstrated SOCS activation in a STAT-independent manner, by recruiting other signaling pathways than Jak/Stat (Paul *et al.*, 2000; He *et al.*, 2003a; Ehlting *et al.*, 2005; Barclay *et al.*, 2007; Yarwood *et al.*, 2008). In this study, no heterozygous SNP was encompassed by the regions amplified for methylation analysis and allele/genotype distribution of *SOCS3* and *SOCS5* variants did not differ between the groups.

SOCS5.1 promoter region was completely unmethylated in all subjects in this study. In our previous study (Ostalska-Nowicka *et al.*, 2011), there was a significantly higher *SOCS5* expression level in SR, but not in SS patients, suggesting an unfavorable outcome with Th_1 phenotype for SR patients. Interestingly, Zhang *et al.* (2013) demonstrated that changes in gene expression level do not have to accompany its aberrant methylation. Thus, it is unlikely for SOCS5 gene to be epigenetically regulated in peripheral blood cells in NS patients and we suppose that other mechanism could be responsible either for up-regulation of this gene in SR patients, or an unknown protective mechanism appears to down-regulate *SOCS5* in PBMCs from SS patients and healthy controls.

Our study has some technical limitations. It would be also recommended to determine if the aberrant SOCS3 methylation is Dnmt-dependent. Nevertheless, here we describe an epigenetic mechanism of *SOCS3* up-regulation in steroid resistant children with nephrotic syndrome, which strongly seems to be regulated by the inflammation process itself, rather than an acquired immune reaction dependent on glucocorticoid action. It requires further studies, though it could be potentially used as an early predictive marker for steroid resistance in NS patients.

Acknowledgements

This study was supported by the Poznan University of Medical Sciences grant for Young Scientists No. 502-14-02229373-09513, in the field of the material collection and all molecular analyses.

The authors declare that they have no competing interests. The authors also declare that the results presented in this paper have not been published previously in whole or in part, except in an abstract format.

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