Genome-Wide Analysis of Histone H3 Lysine 4 Trimethylation in Peripheral Blood Mononuclear Cells of Minimal Change Nephrotic Syndrome Patients

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Key Words

Minimal change nephrotic syndrome • Histone H3 lysine 4 • Trimethylation • Chromatin immunoprecipitation • Microarray

Abstract

Background: Studies of the epigenome have attracted some interest in nephrology. However, to date, our knowledge about the alterations in histone modification in minimal change nephrotic syndrome (MCNS) is unknown. This study aimed to investigate the variations in histone H3 lysine 4 trimethylation (H3K4me3) in peripheral blood mononuclear cells of patients with MCNS. **Methods:** H3K4me3 variations were analyzed in peripheral blood mononuclear cells, from 15 MCNS patients and 15 healthy subjects, using the ChIP-chip approach. ChIP real-time PCR is used to validate the microarray results. In addition, mRNA expression and DNA methylation status can also be further analyzed by quantitative (q) RT-PCR and methyl-DNA immunoprecipitation-q PCR, respectively. **Results:** 848 increased and 231 decreased H3K4me3 probes displaying significant H3K4me3 differences were found in MCNS patients compared with healthy subjects. The results of ChIP real-time PCR coincided well with

the microarray. Expression analysis by qRT-PCR revealed positive correlations between mRNA and H3K4me3 levels. DNA methylation alterations were found on selected positive genes (IL4R, HIVEP3, HPSE2, CDH13 and PRKD2). In addition, we also found that there is an inverse relationship between H3K4me3 and promoter DNA methylation in MCNS patients. **Conclusion:** Our studies indicate that there are significant alterations of H3K4me3 in MCNS patients. These significant H3K4me3 candidates may help to explain the immunological disturbance involved in MCNS patients.

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Introduction

Minimal change nephrotic syndrome (MCNS) is a common glomerular kidney disease in childhood, present in approximately 77% of the large sample of children in the International Study of Kidney Disease in Children [1]. Conversely, among adults, only 20–30% of patients with nephrotic syndrome show this abnormality [2, 3]. MCNS usually responds well to corticosteroid therapy and is considered to be an immunologically mediated disease [4, 5]. Both depressed and enhanced cell-medi-

ated immunity has been observed during relapses. Experimental studies have shown that peripheral blood mononuclear cells (PBMCs) and T cell hybridomas made from patients with MCNS released proteinuria-inducing factors in rats [6–8]. Various cytokines released by PBMCs from MCNS patients have also been demonstrated in vivo and in vitro [9–11]. It has been suggested that these soluble mediators disrupt the glomerular filter, resulting in proteinuria. However, to date, the mechanism of immunological disturbance in MCNS patients is still unknown. Therefore, it is necessary to study the pathogenesis of MCNS further.

Histone lysine methylation, which is one of the important epigenetic modifications, is believed to be part of a histone code and has been implicated in multiple biological processes including gene activation, silencing, X-chromosome inactivation, DNA repair, cell cycle control and DNA methylation [12–14]. Lysines can accept 3 methyl groups and can therefore be monomethylated, dimethylated or trimethylated. Recent evidence suggests that there may be functional differences among these methylation states. Among the various histone lysine methylation patterns, much attention has recently been focused on methylation at lysine 4 of histone H3 (H3K4), owing to its association with active chromatin and gene expression [15–17]. Trimethylated H3K4 (H3K4me3) is preferentially detected at active genes and is proposed to promote gene expression through recognition by transcription-activating effector molecules [18]. Aberrant alterations in histone lysine methylation patterns that change chromatin structure could lead to dysregulated gene transcription and disease progression [13, 19]. Therefore, it is of significant interest to investigate whether there are aberrant H3K4me3 in PBMCs from MCNS patients. This may provide important clues to assist in the development of new treatments for MCNS as well as give us a deeper understanding of the etiology of MCNS.

Recent advances in technology have made it possible to analyze chromatin structure at the genome level in mammalian cells. Genome-wide information on histone modifications can be observed using a technique called chromatin immunoprecipitation linked to microarrays (ChIP-chip), which is unbiased and has high-throughput capability. This new high-throughput strategy has been widely used in epigenetic studies [20–23]. Ren et al. [24] first identified novel targets of the yeast transcription factors Gal4 and Ste12 with ChIP-chip on a yeast intergenic DNA array. Meanwhile, in humans, one of the first ChIP-chip experiments adopted was the use of a CpG island array for screening novel E2F4 targets [25]. Recently, this

Table 1. Characteristics of MCNS patients and healthy controls

Characteristics	MCNS patients (n = 15)	Healthy subjects (n = 15)
Males/females	10/5	10/5
Age, years	41.73 ± 6.12	39.06 ± 6.50
Plasma albumin, g/l	21.20 ± 4.81	43.75 ± 14.38
Proteinuria, g/day	4.45 ± 1.78	negative
Serum creatinine, mg/dl	0.93 ± 0.26	0.96 ± 0.23
Serum cholesterol, mM	7.38 ± 2.35	3.12 ± 1.19

Values are expressed as means ± SD.

approach has been applied successfully to delineate the profile of H3K9 and H3K27 methylation in human disease [26–28].

At present, studies of the epigenome have attracted some interest in nephrology. However, to date, there are no reports of MCNS research from the aspect of histone modification. Hence, in this study, we adopted ChIP-chip technology to profile and compare the variations in H3K4me3 at a genome-wide level of PBMCs from MCNS patients and healthy controls to gain a better understanding of the pathogenic mechanisms in MCNS. Meanwhile, we also explored the relevance of H3K4me3 and DNA methylation under this disease condition.

Materials and Methods

Human Subjects

Fifteen adults with MCNS were enrolled in the study. All the patients had undergone a renal biopsy for an exact histological diagnosis. Diagnostic criteria for nephrotic syndrome were massive proteinuria (>3.5 g/day) and hypoalbuminemia (<3 g/100 ml) with or without edema. These patients were recruited from the inpatient section in the kidney transplantation and hemopurification center of Guilin's 181 Hospital, Guilin. None of these patients were treated with any medications that might have affected our interpretation of the data. Age-, race- and sex-matched healthy controls were recruited through advertising. The clinical features of the patients and healthy subjects are shown in table 1. PBMCs were isolated from heparinized venous blood (10 ml per subject) within 1 h after hemostasis according to the Ficoll-Paque Plus instructions. The ethics committee of the Jinan University approved the study and peripheral blood samples were obtained with the informed consent of all participating individuals.

Isolation of PBMCs

Blood samples were obtained from MCNS patients (n = 15) and healthy donors (n = 15). The blood (10 ml per subject) was diluted with equal volumes of phosphate-buffered saline. An

Table 2. ChIP-qPCR primers used for validation of the CpG array data

Gene	Primer sequence (5' to 3')	Annealing, °C	Size bp
IL4R	F: AGTTCTGTCTAAATGCGTCCGT R: GAACTCAATTCCACCAAACCT	59	290
PRKD2	F: GAGCTGCGAAGGAAGAGGA R: GAGGCTGAGGCACGAGAAT	59	277
HPSE2	F: GGAAAGCATTAGGCGAACAGG R: GCTCGTCCGAATGTAGGGTG	59	143
CDH13	F: GACATTGCCGTCTGTTTCCT R: CCACAGTGCCCTCCTGTTTAG	59	292

F = Forward primer; R = reverse primer.

equal volume of diluted blood was overlaid on Ficoll-Paque Plus in a 1:1 ratio and centrifuged at 800 g for 25 min at 22°C. The PBMC layer was harvested and washed twice with phosphate-buffered saline to remove plasma and Ficoll. These samples were then stored at -80°C until assay.

Chromatin Immunoprecipitation Microarray

The ChIP-chip was performed according to described protocols [29] with some modifications. Briefly, PBMCs were cross-linked with 1% formaldehyde (final concentration) for 10 min at 37°C; glycine (0.125 M) was then added for 5 min at 37°C to stop the reaction. After being washed twice with 10 ml of ice-cold 1 × PBS, the cell pellets were resuspended with 300 µl of lysis buffer (10 mM Tris-HCl, pH 8.0; NaCl 100 mM; EDTA 1 mM, pH 8.0; Na-deoxycholate 0.1%; and protease inhibitors) and incubated on ice for 30 min. Then, the cell suspension was sonicated for a total time of 4 min (30 s 'on' and 30 s 'off') to reduce DNA lengths to 200–1,000 bp. 555 µl of dilution buffer containing a protease inhibitor cocktail was added to each ChIP sample. The lysate was then divided into 3 fractions. The 1st lysate was incubated with anti-K4 trimethylated histone H3 antibody (Upstate Biotechnology, Lake Placid, N.Y., USA) at 4°C overnight. The 2nd lysate was used as an input control and the 3rd lysate was used as a negative control. To collect the immunoprecipitated complexes, 50 µl of magnetic beads (Bangs Laboratories Inc.) were added and incubated for 1 h at 4°C. Pellet beads were prepared by a magnetic separation rack for 2 min at 4°C, with the magnetic beads sequentially washed in low salt (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.1; 150 mM NaCl), high salt (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.1; 500 mM NaCl), LiCl salt (0.25 M LiCl; 1% NP40; 1% deoxycholate; 1 mM EDTA; 10 mM Tris-HCl, pH 8.1) and TE buffers (10 mM Tris-HCl; 1 mM EDTA, pH 8.0). The protein/DNA complexes were eluted and formaldehyde cross-links were reversed by heating the sample at 65°C for 5 h. Samples were treated with RNase for 20 min at 37°C and then with proteinase K overnight. DNA was extracted by the phenol/chloroform method, ethanol-precipitated and resuspended in water. PCR amplification of DNA was carried out with diluted DNA aliquots, according to the whole genome amplification kit (Sigma) instructions. After the amplification, DNA was purified by the

Table 3. Primers used in real-time qRT-PCR

Gene	Primer sequence (5' to 3')	Annealing °C	Size bp
IL4R	F: CAGCACCAAGTGGCACAAC R: TGACATAGCACAACAGGCAGA	59	110
HIVEP3	F: CAGTTTTGGTCATCGGAAGTG R: GGATGTCAGTATTGCCATTG-TATT	59	264
PRKD2	F: CTTCCAGAACAACACGACCAA R: GCATCTCGCCACGAAGTA	59	161
HPSE2	F: GCCTGCCGCTACCGATAA R: TCCTTTGGGATGGATGTGG	59	220

F = Forward primer; R = reverse primer.

QIAquick PCR purification kit (Qiagen), Cy5TM-dUTP and Cy3TM-dUTP-labeled (Invitrogen) methylated K4 precipitated DNA and input DNA, and cohybridized to the human 12K CpG island array (UNH Microarray Centre, Toronto, Ont., Canada). The sequences of the CpG islands on the array and alignment data are available at <http://data.microarrays.ca/>.

The hybridized microarray slides were then scanned using a GenePix 4000B scanner (Axon Instruments, Foster City, Calif., USA). GenePix pro v6.0 was used to read the raw intensity of the image. The resulting text files were imported into Agilent GeneSpring GX software for further analysis. The 2 microarray data sets were normalized in GeneSpring GX using an Agilent 2-color scenario (mainly LOWESS normalization), and then CpGs marked present ('all target values') were chosen for further analysis. The differences between test and control samples were identified by 2-fold change.

Chromatin Immunoprecipitation-Quantitative Polymerase Chain Reaction

ChIP was conducted in the same way as ChIP-chip. The DNA pool from ChIP, input control and negative control was used for quantitative (q) PCR. PCR amplification was performed on an ABI 7700 real-time PCR (Applied Biosystems, Foster City, Calif., USA). The PCR conditions were an initial step of 4 min at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at 59°C and 20 s at 72°C. Primers were designed according to the selected genes for evaluating ChIP on chip data (table 2). To generate a standard curve for each amplicon, threshold cycle (CT) values of serially diluted input DNA, which were extracted in the ChIP experiment, were determined. The H3K4me3 changes were determined using the 2^{-ΔΔCT} method [30]. A melting curve analysis was performed for each reaction to ensure a single peak. Each experiment was performed in triplicate, with the values averaged to obtain 1 datum per sample.

RNA Extraction and Real-Time qRT-PCR

Total RNA was extracted from PBMCs with Trizol reagent (Invitrogen) according to the manufacturer's instructions. The concentration and quality of RNA were measured by UV absorbance at 260 and 280 nm (A260/A280 ratio) and checked by agarose gel

Fig. 1. DNA electrophoretogram. Sonicated effects were judged by the agarose gel electrophoresis. After ultrasonication, the genomic DNA fragments were approximately 1,000 bp. M = DL2000 marker; N = MCNS patients; C = healthy control group.

electrophoresis individually. Two micrograms of total RNA was reverse transcribed into cDNA with M-MLV reverse transcriptase and oligo-dT was used as a primer. Real-time PCR involved SYBR green dye and Taq polymerase. One-tenth of the resulting cDNA template was used for DNA amplification on a 7700 real-time PCR system apparatus. PCR amplification using real-time PCR was performed as described above. A standard curve for each gene was generated by serial dilution of the amplified product standard of the known starting concentration. Glyceraldehyde-3-phosphate dehydrogenase mRNA, which yielded an amplicon of 203 bp, was used as a control (primers 5'AAGAAGGTGGTGAAGCAGGC3' and 5'TCCACCACCCTGTTGCTGTA3') for data normalization. The PCR primers used for each gene in this analysis are given in table 3. Expression was assessed by evaluating CT values. The relative amount of expressed mRNA was calculated by the $2^{-\Delta\Delta CT}$ method [30]. The experiment was performed in triplicate.

Methyl-DNA Immunoprecipitation-qPCR

We prepared genomic DNA from 15 MCNS patients and 15 healthy controls by overnight proteinase K treatment, phenol-chloroform extraction, ethanol precipitation and RNase digestion. Before carrying out methyl-DNA immunoprecipitation, we sonicated genomic DNA to produce random fragments ranging in size from 200 to 1,000 bp. We used 6 μ g of fragmented DNA for a standard methyl-DNA immunoprecipitation assay. Following denaturation (95°C 10 min), the DNA was incubated overnight at 4°C with 8 μ g of 5-methylcytidine monoclonal antibody (Eurogentec). 50 μ l of rabbit anti-IgG magnetic beads (S1430S; BioLabs) were added and incubated for 2 h at 4°C. Magnetic beads-monoclonal antibody-DNA complexes were sequentially washed by gentle mixing at 4°C for 4 min with 1 ml of wash buffer 1 (2 mM EDTA; 20 mM Tris, pH 8.0; 1% Triton X-100; 0.1% SDS; 150 mM NaCl), wash buffer 2 (2 mM EDTA; 20 mM Tris, pH = 8.0; 1% Triton X-100; 0.1% SDS; 500 mM NaCl) and wash buffer 3 (1 mM EDTA; 10 mM Tris, pH = 8.0). After washing the complex-

Table 4. Primers used in methyl-DNA immunoprecipitation-qPCR

Gene	Primer sequence (5' to 3')	Annealing, °C	Size bp
IL4R	F: AGTTCTGTCTAAATGCGTCCGT R: GAACTCAATTCACCAAAACCCT	59	290
HIVEP3	F: CAGCTCGCTATTCTGCTTCG R: CACAGGACCAGTTTGCCATC	59	139
PRKD2	F: GAGCTGCGAAGGAAGAGGA R: GAGGCTGAGGCACGAGAAT	59	277
HPSE2	F: GGAAAGCATTAGGCGAACAGG R: GCTCGTCCGAATGTAGGGTG	59	143
CDH13	F: GACATTGCCGTCTGTTTCCT R: CCACAGTGCCCTCCTGTTTAG	59	292

F = Forward primer; R = reverse primer.

es on a magnetic separation rack for 10 min at 4°C, elution was performed with 400 μ l elution buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 1% SDS). The elution fraction was obtained by phenol-chloroform extraction and ethanol precipitation. The quantity of immunoprecipitated DNA was checked with a Nanodrop spectrophotometer (Agilent). PCR amplification using real-time PCR was performed as described above. Relative enrichment of DNA methylation for each gene was determined by the same method as described above. The PCR primers used for each gene in this analysis are shown in table 4. The experiment was performed in triplicate.

Statistical Analysis

Quantitative data are shown as mean values $\pm$ SD. Statistical analyses were performed using an independent samples t test. $p < 0.05$ was regarded as statistically significant.

Results

Monitoring and Data Analysis by CpG Island Array

In ChIP, the sonicated effects were judged by agarose gel electrophoresis. The genomic DNA fragment was approximately 1,000 bp (fig. 1). To obtain a global overview of the H3K4me3 profiles of the MCNS patients and healthy subjects, the ChIP-chip data were first corrected for background and normalized to remove systematic bias. Methylation profiles were then determined by the ratios between normalized Cy5 and Cy3 intensities. Ratios were used as a cutoff for scoring positive loci for H3K4me3. Hence, we selected the ratios >2 as H3K4me3 targets and identified 1,015 and 389 targets, respective-

Table 5. The 20 selected probes with H3K4me3 alterations between MCNS patients and healthy subjects, identified by ChIP-CpG microarray analysis

Probe ID	Fold change	Within	Genome location	Description
UHNhscpg0001545	13.04	PRKD2	chr19:51892513–51893119	protein kinase D2
UHNhscpg0009117	6.17	ZNF333	chr19:14661327–14662364	ZNF333 protein
UHNhscpg0005260	4.91	TCF3	chr19:1602017–1602493	transcription factor 3
UHNhscpg0007184	3.44	CACNA1D	chr3:53684940–53685411	calcium channel, voltage-dependent, L type
UHNhscpg0001742	2.90	DNMT2	chr10:17282923–17283980	DNA (cytosine-5)-methyltransferase 2
UHNhscpg0009360	2.43	PTPN13	chr4:87734788–87735329	protein tyrosine phosphatase, nonreceptor type
UHNhscpg0007787	2.21	LY75	chr2:160468871–160469279	lymphocyte antigen 75
UHNhscpg0007177	2.17	HIVEP3	chr1:42156111–42156820	HIV type I enhancer
UHNhscpg0008263	2.09	IL4R	chr16:27237494–27237759	interleukin-4 receptor α -chain isoform a
UHNhscpg0011633	2.01	HSPA2	chr14:64076557–64076742	heat shock 70 kDa protein 2
UHNhscpg0000762	0.49	SLC25A15	chr13:40281435–40281946	solute carrier family 25
UHNhscpg0008961	0.43	KIR3DL2	chr19:60055042–60055358	NK receptor
UHNhscpg0003995	0.42	LRP1B	chr2:141707608–141707767	low-density lipoprotein-related protein 1B
UHNhscpg0002047	0.41	MRPL27	chr17:45805214–45805905	mitochondrial ribosomal protein L27 isoform a
UHNhscpg0001251	0.39	PISD	chr22:30387570–30388248	phosphatidylserine decarboxylase
UHNhscpg0002631	0.35	CDH13	chr16:81837847–81838485	cadherin 13 preproprotein
UHNhscpg0009999	0.33	CLNS1A	chr11:77026062–77026447	chloride channel, nucleotide-sensitive protein 1A
UHNhscpg0005112	0.21	KIAA0241	chr7:32911549–32911779	KIAA0241 protein
UHNhscpg0006307	0.22	HPSE2	chr10:100983740–100984257	heparanase 2
UHNhscpg0000148	0.15	METT1OD	chr17:2361322–2361754	MGC3329 protein

ly, using CpG arrays. Next, we analyzed H3K4me3 candidate gene variations between MCNS patients and healthy subjects. Our aim was to identify genes that underwent H3K4me3 changes in MCNS patients compared with healthy subjects. For this, we adopted 2 steps to analyze this data. First, genes that showed significant differences (the ratio >2 or <0.5) between MCNS and control groups were considered H3K4me3 candidates. Second, genes that showed significant methylation differences between MCNS and control groups, but were not identified as H3K4me3 targets, were excluded in the target list. These stringent restrictions might have caused some false negatives (i.e. genes that underwent changes in methylation but were not identified by our analysis), but it reduced the possibility of introducing too many false positives.

Comparison of H3K4me3 Status between MCNS Patients and Healthy Subjects

By applying the above analysis procedure to the CpG array, we found that 1,079 probes displayed significant H3K4me3 differences in MCNS patients compared with healthy subjects. Among these probes, 848 displayed increased H3K4me3, and 231 showed decreased H3K4me3. These identified genes included immunity, cell signal

transduction, protein transcription and synthesis, ion channel and transporters, cell apoptosis, extracellular matrix, etc. The H3K4me3 alterations of the 20 selected genes are presented in table 5.

Validation for CpG Microarray Data

To validate the microarray results, selected genes that displayed increased H3K4me3 (IL4R PRKD2) and decreased H3K4me3 (HPSE2 CDH13) in MCNS patients were then verified by ChIP-qPCR. As shown in table 6, the qPCR results of these chosen H3K4me3 candidates are consistent with the ChIP array analyses. Taken together, these ChIP validations support the accuracy of the array data.

H3K4me3 Alterations and Gene Expression

To confirm correlations between H3K4me3 and gene expression, we performed mRNA expression analysis by real-time qRT-PCR for the 4 randomly selected H3K4me3 candidates (IL4R, HIVEP3, PRKD2 and HPSE2). As shown in table 7, there are mRNA expression changes of the H3K4me3 candidates in MCNS patients compared with healthy subjects. Our results further support the previous views that high levels of H3K4me3 are associated with active genes.

Table 6. Quantitative real-time PCR verification of ChIP-chip data

Probe ID	Gene	Quantitative real-time, PCR		Microarray change
		M	C	
UHNhscpg0008263	IL4R	1.00	1.48 ± 0.03*	U
UHNhscpg0001545	PRKD2	1.00	2.66 ± 0.02*	U
UHNhscpg0006307	HPSE2	1.00	0.79 ± 0.03*	D
UHNhscpg0002631	CDH13	1.00	0.48 ± 0.02*	D

Microarray changes (H3K27me3) are presented as upregulated (U) or downregulated (D) compared to the healthy group. Similarly, real-time PCR values are expressed as the mean ± SD compared to the healthy group. Quantitative data was calculated by $2^{-\Delta\Delta CT}$. Assays were done in triplicate. * $p < 0.05$, which is considered to be statistically significant (independent samples t test).

M = MCNS group; C = healthy subjects group.

Relationships between H3K4me3 and DNA Methylation

To study the mechanisms and the relationships between H3K4me3 and DNA methylation further, we examined the methylation status of the 5 selected positive genes, IL4R, HIVEP3, PRKD2, HPSE2 and CDH13 in MCNS patients compared with healthy subjects. The results are presented in table 8.

Discussion

Modifications of histone tails are thought to specify a code that regulates the expression of genes [31]. The emerging consensus is that high levels of H3K4me3 are associated with active genes. Analyses of H3K4me3 distribution indicate that this histone modification occurs primarily in the vicinity of the transcription start site [32, 33]. To cover these regions in this study, we used human 12K CpG island arrays, which contain a significant percentage of the CpG islands found in the human genome with approximately 68% located near a transcription start site, although not fully representative of promoter regions [34]. H3K4me3 was observed several years back, but its subtle inter-relationships with other epigenetic modifications and potential functional significance in human disease are still not very well known. At present, the consensus is that MCNS involves immune abnor-

Table 7. H3K4me3 variations and mRNA expression levels with real-time qRT-PCR analysis

Probe ID	Gene	qRT-PCR		Microarray change
		M	C	
UHNhscpg0001545	PRKD2	1.00	1.53 ± 0.04*	U
UHNhscpg0008263	IL4R	1.00	1.43 ± 0.03*	U
UHNhscpg0006882	HIVEP3	1.00	1.75 ± 0.02*	U
UHNhscpg0006307	HPSE2	1.00	0.78 ± 0.03*	D
UHNhscpg0002631	CDH13	1.00	0.53 ± 0.02*	D

Microarray changes (H3K4me3) are presented as upregulated (U) or downregulated (D) compared to the healthy group. Similarly, relative mRNA values are expressed as the mean ± SD compared to the healthy group. Quantitative data was calculated by $2^{-\Delta\Delta CT}$. The experiments were done in triplicate. * $p < 0.05$, which is considered to be statistically significant (independent samples t test).

M = MCNS group; C = healthy subject group.

malities in PBMCs including the B and T lymphocytes, monocytes and natural killer cells. Based on these, in this study, we selected H3K4me3 as a target to be investigated by the ChIP-chip strategy and explored the hypothesis that H3K4me3 is associated with the pathogenesis of MCNS.

In the present study, we mainly analyzed the trimethylation status of H3K4 in MCNS patients and healthy subjects. All in all, these genes included genes associated with immunity, cell signal transduction, protein transcription and synthesis, ion channel and transporters, cell apoptosis, DNA and RNA processing and extracellular matrix, etc. Using ChIP-qPCR, we were able to confirm the validity of the microarray data, and the consistency of the results ultimately proves the value of this approach.

Among the candidates identified in the CpG array, we found that IL4R, HIVEP3 and PRKD2 displayed increased H3K4me3. The IL4R gene encodes the α -chain of the interleukin-4 receptor, a type I transmembrane protein that can bind interleukin-4 and interleukin-13 to regulate IgE production [35]. The encoded protein also can bind interleukin-4 to promote differentiation of Th2 cells [36]. A number of reports have shown that MCNS is frequently associated with atopy and elevation of serum IgE levels [5–8]. In this study, we observed that the mRNA expression of IL4R was upregulated, which is consistent with a previous study [37]. HIVEP3, also known as rec-

Table 8. The relationship between H3K27me3 alterations and DNA methylation levels by real-time PCR analysis

Probe ID	Gene	MeDIP-qPCR		Microarray change
		M	C	M
UHNhscpg0008263	IL4R	1.00	0.69 ± 0.02*	U
UHNhscpg0007177	HIVEP3	1.00	0.62 ± 0.05*	U
UHNhscpg0009064	MMP15	1.00	0.83 ± 0.03*	U
UHNhscpg0006307	HPSE2	1.00	1.53 ± 0.02*	D
UHNhscpg0004607	RB1	1.00	1.68 ± 0.02*	D

Microarray changes (H3K4me3) are presented as upregulated (U) or downregulated (D) compared to the healthy group. Similarly, real-time PCR values (DNA methylation levels) are expressed as the mean ± SD compared to the healthy group. Quantitative data was calculated by $2^{-\Delta\Delta CT}$. The experiments were done in triplicate. * $p < 0.05$, which is considered to be statistically significant (independent samples t test).

MeDIP = Methyl-DNA immunoprecipitation; M = MCNS group; C = healthy subjects group.

ognition component (KRC), which is a large protein composed of 2,282 amino acids, is induced upon T cell receptor signaling in T cells and functions to regulate the expression of the interleukin-2 gene. Overexpression of KRC in transformed and primary T cells leads to increased interleukin-2 production, whereas dominant negative KRC, or loss of KRC protein in KRC-null mice, results in a diminished interleukin-2 production [38]. KRC plays a critical role in the NFκB and JNK pathways in regulating CD4 T cell activation, effector function and cytokine gene expression as well as T cell survival and death [39, 40]. PRKD2, a member of the protein kinase D family serine/threonine kinases, is expressed in a wide range of cells and regulates various cellular responses, including immune responses. PRKD2 is involved in interleukin-2 promoter regulation and cell death, depending on its activity upon TCR stimulation [41]. Recently, PRKD2 has also been reported to play a pivotal role in endothelial cell proliferation and migration necessary for angiogenesis at least in part through modulation of the expression of vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1 [42]. In this study, we showed that the mRNA expression of IL4R, HIVEP3 and PRKD2 was upregulated. The H3K4 trimethylation of IL4R, HIVEP3 and PRKD2 will provide a new mechanism for activation of these genes and, therefore, immune abnormalities are involved in MCNS.

We also observed that the other 2 candidate genes, HPSE2 and CDH13, showed a significant decrease in histone H3K4me3 in MCNS patients compared with healthy subjects. HPSE2 is a member of the heparanase family. Heparanase, an endo-β-D-glucuronidase, cleaves heparan sulfate at specific sites, leading to the release of growth factors that may be involved in decidualization and remodeling of the maternal vasculature. It is directly able to modulate the selective permeability of the glomerular capillary wall by its ability to degrade heparan sulfate glycosaminoglycan, which constitutes the charge barrier [43]. CDH13, a member of the cadherin superfamily, which is located at chromosome 16q, is a cell-surface glycoprotein that is responsible for establishing and maintaining intercellular connections. The reduction of H-cadherin expression has been reported in esophageal, breast and lung cancers [44–46]. In this study, our results showed that the mRNA expressions of HPSE2 and CDH13 were downregulated in MCNS patients compared with healthy subjects, and that these might engage in the pathogenesis of MCNS by mechanisms which are unclear at the present.

DNA methylation is a postsynthetic modification that is responsible for epigenetic modulation of gene expression. A large body of work has demonstrated that cytosine methylation of the regulatory sequences of DNA is associated with transcriptional inactivation of genes, while hypomethylation contributes to the activation of transcription [47, 48]. DNA methylation and histone modification may act synergistically or antagonistically on gene expression [22, 31]. Recent studies have suggested that acetylated histones and dimethylated histone H3 at lysine 4 (H3K4me2) are inversely correlated with DNA methylation [49, 50]; however, it is less clear whether increased or decreased H3K4me3 is also accompanied by local changes in DNA methylation in MCNS patients. Thus, we again selected 5 positive genes (IL4, HIVEP3, PRKD2, HPSE2 and CDH13) from this microarray to initially explore whether these modifications coincide in the context of this disease. We found that there is an inverse relationship between H3K4me3 and promoter DNA methylation in MCNS patients. This may indicate that DNA methylation and H3K4me3 are cooperatively involved in the pathogenesis of MCNS, at least in these genes.

We systematically evaluated the status of H3K4me3 in PBMCs of MCNS patients, for the first time, and gained new insights into the links between H3K4me3 and DNA methylation in the context of MCNS. Our results indicate that the alterations of H3K4 trimethylation are associated with the pathogenesis of MCNS and that these novel candidate genes may become potential biomarkers or future

therapeutic targets. Further investigations are needed to clarify the roles of identified H3K4me3 candidate genes in the pathogenesis of MCNS.

Conclusion

MCNS is thought to be a multifactorial disease, and the basic mechanisms behind MCNS are still waiting to be solved. Our results, however, suggest for the first time

that H3K4me3 alterations may play a role in MCNS. Expanding the epigenetic work to the other kidney diseases, therefore, seems called upon.

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