Genome-wide analysis of histone H3 lysine 4 trimethylation by ChIP-chip in peripheral blood mononuclear cells of systemic lupus erythematosus patients

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Abstract

Objective

Histone H3 lysine 4 trimethylation(H3K4me3) is an important epigenetic modification and associated with active transcription in multiple organisms. In systemic lupus erythematosus (SLE), global and gene-specific DNA methylation changes have been demonstrated to occur. However, to date, our knowledge about the alterations in the histone lysine methylation in SLE is known little. This study aimed to investigate the variations in H3K4me3 in CpG island regions in the peripheral blood mononuclear cells (PBMCs) of SLE patients and the controls, including rheumatoid arthritis patients and healthy subjects.

Methods

PBMCs were isolated by density gradient centrifugation from 10 active SLE patients, 7 inactive SLE patients, 8 rheumatoid arthritis patients and 8 healthy volunteers. H3K4me3 variations were analysed by using chromatin immunoprecipitation linked to the microarray (ChIP-chip) approach. ChIP-real time PCR was used to validate the microrray results. Expression analysis by qRT-PCR revealed correlations between mRNA and H3K4me3 levels. In addition, DNA methylation status was also further analysed by Methyl-DNA immunoprecipitation-quantitative PCR.

Results

Many key relevant candidate genes (such as PTPN22, LRP1B etc.) displaying differential changes in H3K4me3 in SLE versus controls (rheumatoid arthritis patients, healthy subjects) were identified. The results of ChIP-real time PCR were coincided well with those of microarray. Aberrant DNA methylation can also be found on selected randomly positive genes (WDR5, SLC24A3, PTPN22, LRP1B METT10D and CDH13).

Conclusions

Our results first indicate that there are significant alterations of H3K4me3 in PBMCs of SLE patients, and H3K4me3 alterations are associated with the pathogenesis of the SLE. Such novel findings show the significance of H3K4me3 as a potential biomarker or promising target for epigenetic-based lupus therapies.

Key words

Systemic lupus erythematosus, histone H3 lysine 4, trimethylation, chromatin immunoprecipitation, rheumatoid arthritis.

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Introduction

Systemic lupus erythematosus (SLE), is a systemic autoimmune disease characterised by the presence of various types of autoantibodies in peripheral blood and a wide array of clinical symptoms. These autoantibodies mediate organ damage by directly binding to host tissues and by forming immune complexes that deposit in vascular tissues and activate cells of the immune system. Despite intensive research, the exact mechanisms of SLE pathogenesis is still unknown and is probably multifactorial. Both genetic and environmental factors have been associated with SLE, but these factors alone are insufficient to explain the onset of SLE. Thus, it is necessary to further study the pathogenesis of SLE.

Histone lysine methylation, which is one of the most important epigenetic modifications, is believed to be part of a histone code (1) and has been implicated in multiple biological processes including gene activation, silencing, X-chromosome inactivation, DNA repair, cell cycle control, and DNA methylation (2-4). Lysine methylation displays the highest degree of complexity among known covalent histone modifications, with each site of methylation regulating the association of different effector molecules (3). Lysines can accept three methyl groups, and therefore can be monomethylated, dimethylated, or trimethylated (5, 6). Recent evidence suggests that there may be functional differences among these methylation states (7). Among the various histone lysine methylation patterns, recently, much attention has focused on methylation at lysine 4 of histone H3(H3K4), owing to its association with active chromatin and gene expression (8-11). H3K4 can be mono-, di- or tri-methylated, Trimethylated H3K4 (H3K4me3) is preferentially detected at active genes and is proposed to promote gene expression through recognition by transcription-activating effector molecules (12). Aberrant alterations in histone lysine methylation patterns that change chromatin structure could lead to dysregulated gene transcription and disease progression (3, 13). Therefore, elucidating the biological and functional relevance of H3K4me3 is crucial to our understanding of the role of chromatin in gene expression. This may provide important clues to assist in the development of new treatments for SLE as well as give us a deeper understanding of the aetiology of this disease. Recent advances in technology have made it possible to analyse chromatin structure at genome-scale 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 an unbiased, high-throughput capability of microarrays. This new high-throughput strategy has been widely used in epigenetic studies (14-17). Ren *et al*. (18) 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 (19). Recently, this approach has been applied successfully to delineate the profile of H3K9 and H3K27 methylation in human disease (20-22).

In recent years, increasing evidence has suggested that epigenetic alterations are related to the pathogenesis of SLE (23), however, most of the evidence about the role of changes in histone modifications in SLE comes from the use of epigenetic drugs and the research limited to only a few of the known gene loci. Hence, in this study, we adopted ChIP-chip technology to profile and compare the variations in H3K4me3 at the genome-wide level of PBMCs from SLE patients and healthy controls, as further better understanding the pathophysiology of SLE. Meanwhile, we also explored the relevance of H3K4me3 and DNA methylation. In addition, we investigated patients with RA, which is a another systemic autoimmune disease, we compared the variations of H3K4me3 expression in SLE patients with those in RA patients and identified changes in H3K4me3 expression that are specific to each condition.

Materials and methods

Human subjects

The study subjects were 8 healthy vol-

unteers, 8 rheumatoid arthritis (RA) patients and 17 SLE patients, of whom 7 had inactive SLE disease and 10 had active SLE disease at the time of the study. All SLE and RA patients were recruited from the outpatient and inpatient in the department of Rheumatology in the Shenzhen People's Hospital, and were not receiving prednisone or other type of immunosuppressive therapy (including cyclophosphamide, methotrexate, azathioprine, and mycophenolate mofetil) at least one month. They are age-, race-, and sex-matched healthy controls were recruited by advertising. All patients fulfilled the American College of Rheumatology criteria for these diseases (24, 25), SLE activity was assessed by the SLE disease activity index (SLEDAI) (26), and those with SLEDAI score ≥5 were considered to have active disease. SLE Patients were classified as active and inactive disease. Relevant information regarding the study subjects are shown in Table I. PBMCs were isolated from heparinised venous blood (10 ml per subject) within 1 hour after hemospasia 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.

Chromatin immunoprecipitation microarray (ChIP-chip)

The ChIP-chip was performed according to described protocols (27, 28) with some modifications. Briefly, PBMCs were crosslinked 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 10ml of ice-cold 1 x PBS, the cell pellets were resuspended with 300 µl Lysis Buffer (10mM Tris-HCl pH 8.0; NaCl 100mM; 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 4 min total time (30s"on" & 30s "off") to reduce DNA lengths to between 200 and 1000 bp. Add 555 µl of dilution buffer containing protease inhibitor cocktail to each ChIP sample. The lysate was then divided into three fractions. The first lysate was incubated with anti-K4 trimethylated histone H3 antibody (from Upstate Biotechnology, Lake Placid, NY) at 4°C overnight. The second lysate was used for input control. The third lysate was used as 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 magnetic separation rack for 2 min at 4°C, then the magnetic beads were sequentially washed in low salt, high salt, LiCl salt, and TE buffers. The protein/DNA complexes were eluted, and formaldehyde crosslinks 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 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 on diluted DNA aliquots, according to the whole genome amplification kit (Sigma) instructions. After the amplification, DNA was purified with the QIAquick PCR purification kit (Qiagen), Cy5™-dUTP and Cy3™-dUTP (Invitrogen) labelled methylated K4 precipitated DNA and input DNA, respectively, and cohybridised (1µg each ChIP/input sample) to the human 12K CpG-island array (UNH Microarray Centre, Toronto, Canada). The sequences of CpG islands on the array and alignment data are available at http://data.microarrays.ca/.

Microarray data collection and statistical analysis of microarray data

The hybridized microarray slides were scanned using a GenePix 4000B scanner (Axon Instruments, Foster City, CA). Acquired microarray images were analyzed with GENEPIX version 6 software. The resulting text files were imported into the Agilent GeneSpring GX software for further analysis. The microarray data sets were normalised in GeneSpring GX using the Agilent Two-color scenario (LOWESS normalisation), and then CpGs marked present ("All Targets Value") were chosen for further analysis. The differences between test and control sample were identified by a 2-fold change (15, 21).

We adopted two steps for analyse these datum. First, genes that show significant differences (the ratio>2 or <0.5) between SLE and control groups were H3K4me3 candidates; second, genes that showed significant methylation differences between SLE 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 not identified by our analysis), but it reduced the possibility of introducing too many false positives.

Chromatin immunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR)

ChIP was conducted the same way as for ChIP-chip. The DNA pool from ChIP, input control and negative control was used for qPCR, PCR amplification was performed on an ABI 7700 Realtime PCR (Applied Biosystems, Foster City, CA).The PCR conditions were an initial step of 4min at 95°C, followed by 40 cycles of 15 s at 95°C,20 s at 59°C and 20s at 72°C. The following primers were used: WDR5, forward 5´-GTCCTCGGTCCTGA ACGGTAGG -3´, reverse 5´- GCAAGCGAGACAGCAGGGAGT-3´; SLC24A3, forward 5´- TGACCGACGATGCTGAGAAA-3´, reverse 5´- TCCATCACCAACGAAACCTG-3´; hfl-B5, forward 5´- CGAGTGGAGTGTCCGCTGT-3´, reverse 5´-GGCACCCGTAGACCGAAG-3; PTPN22,5´-GGA CACTGGATTTTGAAGACTTAGTA, reverse5´-CAGAATGGC AACAGTAGAACAGTG-3´;METT10D, forward 5´-GGGCTAGTGAGGTGCCGACTT-3´, reverse 5´- GCCACTG CCGTTCTTTCCAT-3´; LRP1B, forward 5´-CCCTGGCAATCGGCAATA-3´, reverse 5´-TTACCGGGCGACCAC AAT-3´; CDH13, forward 5´- GACATTGCCGTCTGTTTCCT-3´, reverse 5´-CCACAGTGCCCTCCTGTTTAG-3´.

To generate a standard curve for each amplicon, Ct values of serially diluted input DNA that were extracted in the ChIP experiment were determined. The H3K4me3 changes were determined using the $2^{-\Delta\Delta CT}$ method (29). Melting curve analysis was performed for each

reaction to ensure a single peak. Each experiment was performed in triplicate and the values averaged to obtain one datum per sample.

RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from PBMC with Trizol reagent (Invitrogen) following 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 electrophoresis individually. 2 μg of total RNA was reverse transcribed into cDNA with M-MLV reverse transcriptase and oligo-dT 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 the real-time PCR was performed as described above. A standard curve for each gene was generated by serial dilution of amplified product standard of known starting concentration. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA which yielded an amplicon of 203 bp was used as a control (primers 5′-AAGAAGGTGGTGAAGCAGGC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3') for data normalisation. The PCR primers used for each gene in this analysis are as follows: WDR5 forward 5′- GGAGA AGAAGCCCGAGACCG-3′,reverse 5′-GCAGATGAACTTGCCAGCCAC-3'; SLC24A3,forward 5′-CAAACGA GGATAGAAGACAAGG-3′, reverse 5′-TCCAAGGAAGGGACGAAGA-3′;PTPN22 forward 5′- AACACG-GACC AAATCAACTCCT-3′, reverse 5′- TCATGGTGCTTTACATTAGAG-GC-3′; LRP1B forward 5′-AGCTGT-CAGTTA TGTGATGGCTAC-3′, reverse 5′- TCAGACTTGCTGCTCTTT-GG-3′;CDH13 forward 5′-GGCA-GAACTCGTGA TTGTCG-3′, reverse 5′-GGCTCTTGATCCACTCCCTTT-3′. Expression was assessed by evaluating threshold cycle (CT) values, the relative amount of expressed mRNA was calculated by the $2^{-\Delta\Delta CT}$ method (29). The experiments were done in triplicate.

Methyl-DNA immunoprecipitation-quantitative PCR(MeDIP-qPCR)

We prepared genomic DNA from 10 active SLE, 7 inactive SLE, 8 RA patients and 8 healthy controls respectively by overnight proteinase K treatment, phenol-chloroform extraction, ethanol precipitation and RNase digestion. Before carrying out MeDIP, we sonicated genomic DNA to produce random fragments ranging in size from 200 to 1,000 bp. We used 6 μg of fragmented DNA for a standard MeDIP assay. Following denaturation (95°C 10min), the DNA was incubated overnight at 4°C with 8 μg of 5-methylcytidine monoclonal antibody (Eurogentec). Then 50 μl of Rabbit anti-IgG Magnetic Beads (BioLabs S1430S) was 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[2mM EDTA, 20Mm Tris (pH=8.0), 1% Triton X-100, 0.1% SDS, 150 mM NaCl], wash buffer 2[2mM EDTA, 20Mm Tris (pH=8.0), 1% Triton X-100, 0.1% SDS, 500 mM NaCl] and wash buffer 3[1mM EDTA, 10 Mm Tris (pH=8.0)]. After washing the complexes by magnetic separation rack for 10min at 4°C, then elution was performed with 400μl elution buffer (50mM Tris-HCl, pH=8.0; 10mM EDTA, PH=8.0; 1% SDS). The elution fraction was prepared by phenol-chloroform extraction and ethanol precipitation. The quantity of immuno-precipitated DNA was checked with Nanodrop spectrophotometer (Agilent). PCR amplification using the real-time PCR was performed as described above. Relative enrichment of DNA methylation for each gene was determined by the same method described above. The PCR primers used for each gene in this analysis are as follows: WDR5 forward 5′- GGTGCTT-TCCAAATGCCCACT-3′, reverse 5′-CAGGCCCTTCTAATGCACTTC-CTA-3′; SLC24A3 forward 5′- TGCT-GCTCTGGTCGCTGTCG-3′, reverse 5′-CCCACCTCCATGCCTCTGC-3′; PTPN22 forward 5′-CTAAAAATA-CAGCGGTGAACAAA-3′, reverse 5′-CTTTTTACATCTATGCCTT-GGGT-3′; LRP1B forward 5′-ACCCT-GGCAATCGGCAATAA-3′, reverse 5′-CCCTCCACCTTCCACATCCTG-3′; METT10D forward 5′-TTGAA-GAAAGAAGAAACCCTCG-3′, reverse 5′-TGTCCTCCTCCTGCCAT-ACC-3′. CDH13 forward 5′-GACATT-GCCGTCTGTTTCCT-3′, reverse 5′-CCACAGTGCCCTCCTGTTTAG-3'. The experiments were done in triplicate.

Statistical analysis of quantitative PCR

Quantitative data are shown as mean values ± SE. Statistical analyses were performed using one-way ANOVA. $p<0.05$ was regarded as statistically significant.

Results

To obtain a global overview of H3K4me3 profiles of active SLE, inactive SLE, RA patients and healthy controls, the ChIP-chip data were first corrected for background and normalised to remove systematic bias. Methylation profiles were then determined by the ratios between normalised Cy5 and Cy3 intensities. The ratio >2 was used as a cut-off for scoring positive for H3K4me3 (15, 21). Hence, we selected the ratio >2 as H3K4me3 targets and identified 271, 224, 129 and 489 targets, respectively, using CpG arrays. Detailed lists of candidate genes are provided in Supplemental Tables S1-S4. Then, we analysed H3K4me3 candidate gene variations between SLE patients (active SLE, inactive SLE) and controls (RA patients and healthy subjects).

In this study, we identified many genes displayed significant H3K4me3 differences in SLE patients compared with controls. These identified genes included immunity, cell signal transduction, protein transcription and synthesis, ion channel and transporters, cell apoptosis, DNA and RNA processing, and extracellular matrix etc. Since there are many significant H3K4me3 difference genes referring to these ways, we selected one or two representative genes ($p<0.05$) for each category. At the same time, we also selected some genes, confirmed by some literature, which are associated with the pathogenesis of SLE. Finally, we respectively selected 20 genes with significant H3K4me3

Table I. Demographics, disease manifestations in the SLE patients and control subjects.

Characteristic	SLE patients		RA patients (*n*=8)	Healthy controls (*n*=8)
	Active	Inactive		
NO.Female/male	10/0	7/0	8/0	8/0
Age mean (range) years	35.7 (20-56)	39.9 (20-60)	43.1 (33-67)	35 (24-57)
Race	Asian	Asian	Asian	Asian
SLEDAI score	11.6 (6-16)	2.0 (0-4)	N/A*	N/A*
Disease manifestations				
Skin rash	4	0	0	0
Mucosal ulcers	2	0	0	0
Alopecia	2	0	0	0
Proteinuria	5	1	0	0
Hematuria	1	0	0	0
Pyuria	4	1	0	0
Vasculitis	1	0	0	0
Arthritis	3	0	8	0
Low complement	8	2	0	0
Increased dsDNA	8	1	0	0
Pericarditis	1	0	0	0
Leukopenia	1	0	0	0

N/A*: not applicable.

differences for making following Table II-VII.

Comparison of H3K4me3 status between active SLE and healthy subjects

By applying the above analysis procedure to the CpG array, we found that 413 probes displayed significant H3K4me3 differences in active SLE compared with healthy subjects. Among these probes, 137 probes displayed increased H3K4me3 and 276 decreased H3K4me3. Particularly, we found that WD repeat domain 5(WDR5), which is a conserved subunit of Trithorax (TRX) histone methyltransferase complexes, displayed increased H3K4me3. Recent studies have shown that it is involved in di- to trimethylation conversion of histone H3 at K4 (30, 31). The H3K4 me3 alterations of selected 20 genes are presented in Table II. Detailed lists of genes with H3K4me3 alterations are provided in Supplemental Table S5.

Comparison of H3K4me3 status between inactive SLE and healthy subjects

We took the same analysis procedure to the CpG array and identified 393 probes with significant H3K4me3 differences in inactive SLE compared with healthy subjects. Among these probes, 112 displayed increased H3K4me3 and 281 decreased H3K4me3. The H3K4me3 alterations of selected 20 probes are presented in Table III. Detailed lists of genes with H3K4me3 alterations are provided in Supplemental Table S6.

Comparison of H3K4me3 status between active and inactive SLE patients

We made a comparison of H3K4me3 alterations between active and inactive SLE patients and identified 270 probes with significant H3K4me3 differences. Among these probes, 151 displayed increased H3K4me3 and 119 showed decreased H3K4me3. The selected 20 probes with H3K4me3 alterations are presented in Table IV. Detailed lists of genes with such alterations are provided in Supplemental Table S7.

Table II. The selected 20 probes with H3K4me3 alterations between active SLE and healthy subjects, identified by ChIP-CpG microarray analysis.

Probe ID	Fold change	Within	Genome location	Description
UHNhscpg0003698	4.93	PTPN13	chr4:87904592-87904686	protein tyrosine phosphatase,non-recepter type
UHNhscpg0002978	4.26	HPSE2	chr10:100809507-100809589	heparanase2
UHNhscpg0002961	4.15	QRSL1	chr6:107184320-107184857	gltaminyl-tRNA synthase
UHNhscpg0005325	3.40	JAM2	chr21:25980859-25980957	junctional adhesion molecule 2 precursor
UHNhscpg0001094	3.40	SLC24A3	chr20:19621353-19621707	solute carrier family 24
UHNhscpg0008640	3.13	TRPC6	chr11:100958085-100959004	transient receptor potential cation channel
UHNhscpg0009361	2.75	hfl-B5	chr11:32561673-32562466	B5 receptor
UHNhscpg0000081	2.16	LACTB2	chr8:71743478-71744294	lactamase, beta 2
UHNhscpg0010826	2.01	NF704	chr8:81761935-81762442	zinc finger protein 704
UHNhscpg0011173	2.00	WDR5	chr9:135990906-135991845	WD repeat domain 5
UHNhscpg0000213	0.49	C6orf170	chr6:121697099-121697968	protein C6orf170
UHNhscpg0002836	0.47	GABRB3	chr15:24567820-24568224	gamma-aminobutyric acid A receptor, beta
UHNhscpg0004001	0.38	RGS13	chr1:190890876-190891120	regulator of G-protein signalling 13
UHNhscpg0003995	0.38	LRP1B	chr2:141707608-141707767	low density lipoprotein-related protein 1B
UHNhscpg0002631	0.35	CDH13	chr16:81837847-81838485	cadherin 13 preproprotein
UHNhscpg0000148	0.35	METT10D	chr17:2361322-2361754	MGC3329 protein.
UHNhscpg0007357	0.34	TMSB10	chr2:84986322-84986645	hymosin, beta 10
UHNhscpg0008961	0.31	KIR3DL2	chr19:60055042-60055358	NK receptor.
UHNhscpg0005333	0.23	PTPN22	chr1:114201225-114201328	protein tyrosine phosphatase,non-recepter type
UHNhscpg0000607	0.22	PVRL3	chr3:112274059-112274383	PVRL3 protein.

Table III. The selected 20 probes with H3K4me3 alterations between inactive SLE and healthy subjects, identified by ChIP-CpG microarray analysis.

Probe ID	Fold change	Within	Genome location	Description
UHNhscpg0010529	7.02	GI2	chr4:24625819-24625958	leucine-rich repeat LGI family, member 2
UHNhscpg0004072	5.92	PDE7B	chr6:136416011-136416273	phosphodiesterase 7B
UHNhscpg0000511	3.37	HLA-E	chr3:112274059-112274383	PVRL3 protein.
UHNhscpg0004463	3.18	HLA-E	chr6:30565074-3056537	major histocompatibility complex,class I,E
UHNhscpg0001094	2.96	SLC24A3	chr20:19621353-19621707	solute carrier family 24
UHNhscpg0002314	2.52	KCNAB1	chr3:157491665-157492083	potassium voltage-gated channel, shake-related
UHNhscpg0008425	2.41	RPSA	chr3:39422803-39423639	ribosomal protein SA
UHNhscpg0007005	2.21	GRIA1	chr5:152946175-152946339	glutamate receptor, ionotropic
UHNhscpg0011144	2.20	LARS2	chr3:45415147-45415391	leucyl-tRNA synthetase 2
UHNhscpg0004823	2.02	RASA2	chr3:142739807-142739862	RAS p21 protein activator 2
UHNhscpg0000999	0.47	FLJ11193	chr5:31579621-31579975	FLJ11193 protein.
UHNhscpg0000148	0.43	METT10D	chr17:2361322-2361754	MGC3329 protein.
UHNhscpg0000653	0.41	BC011600	chr6:31973515-31974151	RD RNA binding protein.
UHNhscpg0003995	0.38	LRP1B	chr2:141707608-141707767	low density lipoprotein-related protein 1B
UHNhscpg0009599	0.37	SATB2	chr2:200031715-200032334	SATB family member 2
UHNhscpg0007357	0.34	TMSB10	chr2:84986322-84986645	thymosin, beta 10
UHNhscpg0002631	0.34	CDH13	chr16:81837847-81838485	cadherin 13 preproprotein
UHNhscpg0005333	0.32	PTPN22	chr1:114201225-114201328	protein tyrosine phosphatase,non-recepter type
UHNhscpg0001574	0.27	SF3B5	chr6:144458001-144458742	SF3b10
UHNhscpg0004001	0.26	RGS13	chr1:190890876-190891120	regulator of G-protein signalling 13

Table IV. The selected 20 probes with H3K4me3 alterations between active and inactive SLE, identified by ChIP- CpG microarray analysis.

Probe ID	Fold change	Within	Genome location	Description
UHNhscpg0000061	4.95	PTPN4	chr2:120233974-120234860	protein tyrosine phosphatase, non-recepter type
UHNhscpg0003442	3.94	TRPM2	chr21:44619534-44619975	transient receptor potential cation channel
UHNhscpg0011173	3.62	WDR5	chr9:135990906-135991845	WD repeat domain 5
UHNhscpg0010072	3.23	ABLIM3	chr5:148500613-148501795	actin binding LIM protein family, member 3
UHNhscpg0005029	2.71	AAA1	chr7:34395585-34396352	AAA1 protein isoform V
UHNhscpg0002264	2.70	CDH13	chr16:81837847-81838485	cadherin 13 preproprotein
UHNhscpg0005887	2.68	TRIM2	chr4:154445764-154445897	tripartite motif-containing 2
UHNhscpg0000200	2.61	CASC3	chr17:35549758-35551054	cancer susceptibility candidate 3
UHNhscpg0002961	2.37	QRSL1	chr6:107184320-107184857	glutaminyl-tRNA synthase
UHNhscpg0001860	2.13	C1orf73	chr1:210274732-210276110	C1orf73 protein
UHNhscpg0003976	0.49	PDE7B	chr6:136416011-136416273	phosphodiesterase 7B
UHNhscpg0007129	0.47	MPP2	chr17:39340273-39340455	palmitoylated membrane protein 2
UHNhscpg0007784	0.47	XP07	chr8:21917232-21917561	exportin 7
UHNhscpg0000213	0.43	C6orf170	chr6:121697099-121697968	protein C6orf170
UHNhscpg0004176	0.41	PGBD3	chr10:50417202-50417355	piggyBac transposable element derived 3
UHNhscpg0004466	0.38	BMP4	chr14:53492075-53492857	bone morphogenetic protein 4 preproprotein
UHNhscpg0011618	0.36	PCCB	chr3:137495499-137495597	propionyl coenzyme A carboxylase
UHNhscpg0000511	0.35	PVRL3	chr3:112274059-112274383	PVRL3 protein
UHNhscpg0001296	0.31	FLG25439	chr5:34874723-34875441	FLJ25439 protein
UHNhscpg0006611	0.26	GRIA4	chr11:104985849-104986084	GRIA4 protein

Comparison of H3K4me3 status between active SLE and RA patients

In our study, using CpG island microarray analyses, we also made a comparison on the level of H3K4me3 in the patients with active SLE and RA patients.236 H3K4me3 candidate genes were found to best discriminate the two groups. Among these genes, 169 displayed increased H3K4me3 and 67 showed decreased H3K4me3, the selected 20 probes with H3K4me3 alterations are presented in Table V. Detailed lists of genes with such alterations are provided in Supplemental Table S8.

Comparison of H3K4me3 status between inactive SLE and RA patients

As the same above, we made a comparison of H3K4me3 alterations between inactive SLE and RA patients, and found that 202 probes with significant H3K4me3 differences. Among these probes, 139 displayed increased H3K4me3 and 63

Table V. The selected 20 probes with H3K4me3 alterations between active SLE and RA, identified by ChIP-CpG microarray analysis.

Probe ID	Fold change	Within	Genome location	Description
UHNhscpg0000061	6.29	PTPN4	chr2:120233974-120234860	protein tyrosine phosphatase,non recepter type
UHNhscpg0000081	4.09	LACTB2	chr8:71743478-71744294	lactamase, beta 2
UHNhscpg0002492	3.74	CENTG2	chr2:236606828-236607245	centaurin, gamma 2 isoform 1
UHNhscpg0005029	3.38	AAA1	chr7:34395585-34396352	AAA1 protein isoform V
UHNhscpg0007503	2.84	UGT3A1	chr5:36026368-36026581	hypothetical protein FLJ34658
UHNhscpg0000200	2.47	CASC3	chr17:35549758-35551054	cancer susceptibility candidate 3
UHNhscpg0001860	2.42	C1orf73	chr1:210274732-210276110	C1orf73 protein.
UHNhscpg0002631	2.41	CDH13	chr16:81837847-81838485	cadherin 13 preproprotein
UHNhscpg0003182	2.33	LEMD3	chr12:63850471-63850719	LEM domain containing 3
UHNhscpg0001094	2.33	SLC24A3	chr20:19621353-19621707	solute carrier family 24
UHNhscpg0006834	0.47		chr1:227321939-227322101	
UHNhscpg0001005	0.44		chr16:16476015-16476521	
UHNhscpg0000148	0.42	METT10D	chr17:2361322-2361754	MGC3329 protein.
UHNhscpg0006761	0.38		chr13:19632469-19632710	
UHNhscpg0000102	0.33		chr2:174916344-174917527	
UHNhscpg0010988	0.33	TRIM7	chr5:180562969-180563716	tripartite motif-containing 7 isoform 1
UHNhscpg0005352	0.32	C15orf24	chr15:32181099-32181524	chromosome 15 open reading frame24
UHNhscpg0002293	0.24	LHX9	chr1:196151970-196152540	LIM homeobox 9 isoform 2
UHNhscpg0003862	0.19	KDELR2	chr7:6471766-6471934	KDEL receptor 2
UHNhscpg0000166	0.18	SLC12A8	chr3:126414031-126414955	solute carrier family 12 member 8

Table VI. The selected 20 probes with H3K4me3 alterations between inactive SLE and RA, identified by ChIP-CpG microarray analysis.

Probe ID	Fold change	Within	Genome location	Description
UHNhscpg0010529	5.18	LGI2	chr4:24625819-24625958	leucine-rich repeat LGI family, member 2
UHNhscpg0000009	4.55	RERE	chr1:8799536-8800122	atrophin-1 like protein
UHNhscpg0000213	4.00	C6orf170	chr6:121697099-121697968	protein C6orf170
UHNhscpg0000081	3.00	LACTB2	chr8:71743478-71744294	lactamase, beta 2
UHNhscpg0000511	2.57	PVRL3	chr3:112274059-112274383	PVRL3 protein
UHNhscpg0003657	2.53	ZNF195	chr11:3340253-3340295	zinc finger protein 195
UHNhscpg0002631	2.29	CDH13	chr16:81837847-81838485	cadherin 13 preproprotein
UHNhscpg0001876	2.19	PRR3	chr6:30638118-30638662	proline rich 3
UHNhscpg0011144	2.07	LARS2	chr3:45415147-45415391	leucyl-tRNA synthetase 2
UHNhscpg0006282	2.03	SAV1	chr14:50203675-50203889	WW45 protein
UHNhscpg0010767	0.47	ESD	chr13:46268526-46268875	esterase D
UHNhscpg0001748	0.46		chr15:82539899-82540692	
UHNhscpg0007681	0.45	TBC1D5	chr3:17464432-17464485	TBC1 domain family, member 5
UHNhscpg0001005	0.36		chr16:16476015-16476521	
UHNhscpg0005159	0.33	BC005991	chr6:100069473-100070296	SP45 protein.
UHNhscpg0006946	0.32		chrY:10646218-10646504	
UHNhscpg0001092	0.30	DDX1	chr2:15649013-15649865	DEAD box polypeptide 1
UHNhscpg0009160	0.21	FSHR	chr2:49130887-49131127	follicle stimulating hormone receptor isoform 1
UHNhscpg0001975	0.12	MOV10L1	chr22:48892370-48893581	MOV10-like 1
UHNhscpg0004019	0.09	PAX3	chr2:222868477-222868620	paired box gene 3 isoform PAX3e

showed decreased H3K4me3. The selected 20 probes with H3K4me3 alterations are presented in Table VI. Detailed lists of genes with such alterations are provided in Supplemental Table S9.

Comparison of H3K4me3 status between RA patients and healthy subjects

At the present study, we also made a comparison of H3K4me3 alterations between RA patients and healthy subjects, and found that 364 probes with significant H3K4me3 differences. Among these probes, 66 displayed increased H3K4me3 and 298 showed decreased H3K4me3. The selected 20 probes with H3K4me3 alterations are presented in Table VII. Detailed lists of genes with such alterations are provided in Supplemental Table S10.

Validation for CpG microarray data

To validate the microarray results, selected genes that displayed increased H3K4me3 (WDR5 SLC24A3 hfl-B5) and decreased H3K4me3 (PTPN22 METT10D LRP1B CDH13) in SLE patients were then verified by ChIP-qPCR. As shown in Table VIII, the qPCR results of these chosen K4me3 candidates are consistent with the ChIP array analyses.

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

ProbeID	Fold change	Within	Genome location	Description
UHNhscpg0000166	17.76	SLE12A8	chr3:126414031-126414955	solute carrier family 12
UHNhscpg0003862	6.62	KDELR2	chr7:6471766-6471934	KDEL receptor 2
UHNhscpg0004019	4.77	PAX3	chr2:222868477-222868620	paired box gene 3 isoform PAX3e
UHNhscpg0001092	3.50	DDX1	chr2:15649013-15649865	DEAD(Asp-Glu-Ala-Asp)box polypeptide 1
UHNhscpg0005352	3.39	C15orf24	chr15:32181099-32181524	chromosome 15 open reading frame 24
UHNhscpg0002293	3.13	LHX9	chr1:196151970-196152540	LIM homeobox 9 isoform 2
UHNhscpg0009144	2.68		chr3:10027888-10028179	
UHNhscpg0007985	2.53	TMEM16B	chr12:5862530-5862631	transmembrane protein 16B
UHNhscpg0011502	2.35		chr11:59075092-59075158	
UHNhscpg0010767	2.35	ESD	chr13:46268526-46268875	esterase D
UHNhscpg0001181	0.49	ZNF225	chr19: 49308929-49309510	zinc finger protein 225
UHNhscpg0000999	0.46	FLJ11193	chr5:31579621-31579975	FLJ11193 protein
UHNhscpg0008961	0.43	KIR3DL2	chr19:60055042-60055358	NK receptor
UHNhscpg0004001	0.30	RGS13	chr1:190890876-190891120	regulator of G-protein signalling 13
UHNhscpg0009599	0.31	SATB2	chr2:200031715-200032334	SATB family member 2
UHNhscpg0005333	0.31	PTPN22	chr1:114201225-114201328	protein tyrosine phosphatase,non-recepter type
UHNhscpg0001043	0.29	ZNF222	chr19:49220645-49221654	zinc finger protein 222
UHNhscpg0003182	0.21	LEMD3	chr12:63850471-63850719	LEM domain containing 3
UHNhscpg0000009	0.20	RERE	chr1:8799536-8800122	atrophin-1 like protein
UHNhscpg0002631	0.15	CDH13	chr16:81837847-81838485	cadherin 13 preproprotein

Taken together, these ChIP validations support the accuracy of the array data.

The correlations between H3K4me3 and gene expression

To confirm correlations between H3K4me3 and gene expression, we next performed mRNA expression analysis by real-time quantitative RT-PCR for the five randomly selected H3K4me3 candidates (WDR5 SLC24A3 PTPN22 LRP1B, CDH13). As shown in Table IX, H3K4me3 variations in SLE patients compared with healthy controls have a positive correlation with mRNA expression levels.

The relationships between H3K4me3 and DNA methylation

To further study the mechanisms and the relationships between H3K4me3 and DNA methylation, we next examined the methylation status of the selected five positive genes, WDR5, SLC24A3, PTPN22, LRP1B, METT10D and CDH13 in SLE patients compared with healthy controls, the results are presented in Table X.

Discussion

Modifications of histone tails are thought to specify a code that regulates the expression of genes (32). The emerging consensus is that high levels of H3K4 trimethylation are associated with active genes. Analyses of the H3K4me3 distribution indicate that this histone modification occurs primarily in the vicinity of the transcription start site (10, 33, 34). For cover these regions, in this study, we used human 12K CpG island arrays, which contains a significant percentage of the CpG islands found in the human genome with ~ 68% located near a transcription start site, although not fully representative of promoter regions (35). H3K4me3 has been observed several years back, but its subtle interrelationships with other epigenetic modifications and potential functional significance are still known very little in human disease. At the present, the consensus is that SLE involves immune abnormalities in PBMCs include B and T lymphocyte, monocytes, and natural killer cells. Based on these, we selected H3K4me3 as a target investigated by ChIP-chip strategy and explored the hypothesis that H3K4me3 are associated with the pathogenesis of SLE.

We mainly analysed the trimethylation status of H3K4 in PBMCs of active and inactive SLE patients. To identify genes that underwent changes in H3K4me3 status in SLE patients, RA patients and healthy subjects were chosen as controls. We made a comparison among active SLE, inactive SLE, RA patients and healthy subjects. Subsequently, we identified key relevant candidate genes displaying differential changes in H3K4me3 in SLE versus controls. 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. The changes of H3K4me3 between active and inactive SLE patients might be correlated with the degree of disease severity. Using ChIP-qPCR, we were able to confirm the validity of the microarray data, and the identification of selected genes ultimately proved the value of this approach.

Among the candidates identified in the CpG array, interestingly, we found that WD repeat domain 5(WDR5) displayed increased H3K4me3 between active SLE and healthy controls. WDR5 is a conserved subunit of Trithorax (TRX) histone methyltransferase complexes which is required for recruiting the methyltransferase complexes to the K4-dimethylated H3 tail and is involved

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

Probe ID	Gene	Quantitative real time PCR				Microarray change		
		C (n=8)	RA (n=8)	AS (n=10)	IS (n=7)	RA	AS	IS
UHNhscpg0011173	WDR5	1.00	1.07 ± 0.03	1.39 ± 0.02*	0.99 ± 0.01	NC	U	NC
UHNhscpg0001094	SLC24A3	1.00	1.08 ± 0.03	1.69 ± 0.02*	1.13 ± 0.03*	NC	U	U
UHNhscpg0009361	hfl-B5	1.00	1.05 ± 0.04	1.78 ± 0.03*	1.08 ± 0.03	NC	U	NC
UHNhscpg0005333	PTPN22	1.00	0.76 ± 0.03*	0.70 ± 0.02*	0.78 ± 0.04*	D	D	D
UHNhscpg0000148	METT10D	1.00	0.94 ± 0.05	0.21 ± 0.01*	0.24 ± 0.01*	NC	D	D
UHNhscpg0003995	LRP1B	1.00	0.96 ± 0.03	0.29 ± 0.01*	0.35 ± 0.03*	NC	D	D
UHNhscpg0002631	CDH13	1.00	0.54 ± 0.04*	0.31 ± 0.01*	0.43 ± 0.02*	D	D	D

C: Healthy control group; RA: Rheumatoid arthritis group; AS: Active SLE group; IS: Inactive SLE group. Microarray changes (H3K4me3) are presented as upregulated (U), downregulated (D), no change (NC) compared to the healthy control group. Similarly, real time PCR values were expressed as mean ± SE compared to the healthy control group. Quantitative data were calculated by $2^{-\Delta\Delta CT}$. A single asterisk [*] indicates $p<0.05$,which was considered statistically significant (one-way ANOVA). The assays were done in triplicate.

Table IX. Correlations between H3K4me3 variations and mRNA expression levels by real-time quantitative RT-PCR analysis.

Probe ID	Gene	qRT-PCR				Microarray change		
		C (n=8)	RA (n=8)	AS (n=10)	IS (n=7)	RA	AS	IS
UHNhscpg0011173	WDR5	1.00	1.24 ± 0.02*	2.02 ± 0.03*	1.11 ± 0.01*	NC	U	NC
UHNhscpg0001094	SLC24A3	1.00	1.31 ± 0.03*	1.92 ± 0.02*	1.52 ± 0.03*	NC	U	U
UHNhscpg0005333	PTPN22	1.00	0.54 ± 0.02*	0.55 ± 0.01*	0.59 ± 0.02*	D	D	D
UHNhscpg0003995	LRP1B	1.00	0.50 ± 0.03*	0.40 ± 0.02*	0.40 ± 0.01*	NC	D	D
UHNhscpg0002631	CDH13	1.00	0.41 ± 0.01*	0.48 ± 0.03*	0.46 ± 0.03*	D	D	D

C: Healthy control group; RA: Rheumatoid arthritis group; AS: Active SLE group; IS: Inactive SLE group. Microarray changes(H3K4me3)are presented as upregulated (U), downregulated (D), no change (NC) compared to the healthy control group. Similarly, relative mRNA values were expressed as mean ± SE compared to the healthy control group. Quantitative data were calculated by $2^{-\Delta\Delta CT}$. A single asterisk [*] indicates $p<0.05$,which was considered statistically significant (one-way ANOVA). The assays were done in triplicate.

Table X. The relationship between H3K4me3 alterations and DNA methylation levels with real time PCR analysis.

Probe ID	Gene	MeDIP-qPCR				Microarray change		
		C (n=8)	RA (n=8)	AS (n=10)	IS (n=7)	RA	AS	IS
UHNhscpg0011173	WDR5	1.00	0.92 ± 0.01*	0.88 ± 0.02*	1.35 ± 0.02*	NC	U	NC
UHNhscpg0001094	SLC24A3	1.00	0.60 ± 0.01*	0.43 ± 0.01*	0.48 ± 0.01*	NC	U	UD
UHNhscpg0005333	PTPN22	1.00	1.11 ± 0.02*	1.43 ± 0.01*	2.34 ± 0.02*	D	D	D
UHNhscpg0003995	LRP1B	1.00	1.44 ± 0.01*	1.87 ± 0.01*	1.13 ± 0.01*	NC	D	D
UHNhscpg0000148	METT10D	1.00	1.22 ± 0.02*	1.30 ± 0.01*	1.55 ± 0.02*	NC	D	D
UHNhscpg0002631	CDH13	1.00	1.32 ± 0.01*	1.48 ± 0.02*	1.21 ± 0.02*	D	D	D

C: Healthy control group; RA: Rheumatoid arthritis group; AS: Active SLE group; IS: Inactive SLE group. Microarray changes(H3K4me3) are presented as upregulated (U), downregulated (D), no change (NC) compared to the healthy control group. Similarly, real time PCR values (DNA methylation levels) were expressed as mean ± SE compared to the healthy control group. Quantitative data were calculated by $2^{-\Delta\Delta CT}$. A single asterisk [*] indicates $p<0.05$, which was considered statistically significant (one-way ANOVA). The assays were done in triplicate.

in di- to trimethylation conversion of histone H3 at K4 (30, 31). WDR5 can potentially augment gene expression by increasing K4 trimethylation, because K4me3 is usually a gene activation mark. However, we also found that WDR5, whose gene expression was higher despite not showing significant changes in H3K4me3 in inactive SLE and RA patients compared with healthy controls respectively. To explain the phenomenon observed, we made a further analysis of DNA methylation status in this gene. As anticipated, WDR5 displayed DNA hypomethylation in RA patients, but surprisingly, WDR5 displayed DNA hypermethylation in inactive SLE patients. This exception is probably due to the fact that there are other histone modifications with transcriptional activation in this gene to antagonist gene silencing results from DNA hypermethylation. Our results will further support previous theories that one gene locus may have multiple epigenetic modifications, and the total sum of these modifications may be the

ultimate determinant of the chromatin state, which governs gene transcriptional activation or repression. At the same time, the results also underscore the complexity of methylation regulation.

We also observed that the other two candidate genes PTPN22 and LRP1B showed a significant decrease in histone H3K4me3 in active and inactive SLE patients compared with healthy subjects, respectively. The gene protein tyrosine phosphatase, non-receptor type 22(PTPN22) is located on chromosome 1p13.3-p13.1 and encodes an important negative regulator of T-cell activation, called the lymphoid-specific phosphatase (Lyp) (36, 37), Lyp is expressed in lymphocytes, where it physically associates through its proline-rich motif (called P1) with the SH3 domain of the protein tyrosine kinase Csk, an important suppressor of the Src family of kinases Lck and Fyn, which mediate TCR signalling and is thought to inhibit T-cell activation through its association with the Csk tyrosine kinase (36, 38). PTPN22 has been shown to be associated with SLE, RA, type1 diabetes, and other autoimmune disorders (37, 39-41). In this study, the mRNA expression of PTPN22 was downregulated. This maybe produce inversely cascade reactions as described above and lead to T-cell activation at last. Our novel new observation (decreased H3K4me3 in PTPN22) maybe provide a new mechanism for explaining activation of T-cells in SLE patients. The histone H3K4me3 alteration of PTPN22 is also found between RA patients and healthy controls, this indicates that decreased H3K4me3 in PTPN22 might be correlated with autoimmune diseases. For the low density lipoprotein (LDL) receptor-related protein 1B (LRP1B) gene, Our results showed that the mRNA expression of LRP1B was downregulated in active and inactive SLE patients compared with healthy subjects, respectively, LRP1B is a newly identified member of the LDL receptor family. Its overall domain structure and large size (approximately 600 kDa) are similar to LRP and suggest that it is a multifunctional cell surface receptor (42). It might provide a novel potential explanation for increased atherosclerosis in lupus. In addition, we also observed an exception that the mRNA expression of LRP1B was lower despite did not show significant changes in H3K4me3 in RA patients compared with healthy controls. Similarly, we also made further analysis of DNA methylation status and found LRP1B displayed hypermethylation. The result better explain for this exception.

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 (43, 44). DNA methylation and histone modification may act synergistically or antagonistically on gene expression (15, 32). Recent studies have suggested that acetylated histones and dimethylated histone H3 at lysine 4(H3K4me2) are inversely correlated with DNA methylation (45, 46), however, less clear is whether increased or decreased H3K4me3 is also accompanied by local changes in DNA methylation in SLE patients. Thus, here we again selected five positive genes (WDR5 SLC24A3 METT10D PTPN22 LRP1B CDH13) from this microarray to initially explore whether these modifications coincide in the context of this disease. We found that there are an inverse relationship between H3K4me3 and promoter DNA methylation in SLE patients. This may indicate that DNA methylation and H3K4me3 are cooperatively involved in the pathogenesis of SLE, at least in these genes.

Taken together, here, for the first time, we systematically evaluated the status of H3K4me3 in PBMCs of SLE patients and gained new insights into the links between key genes and histone methylation in the context of SLE. Our results indicate that H3K4 trimethylation involved in the pathogenesis of SLE and 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 SLE.

Supplementary data

The Supplemental Tables S1-S10 are available at *Clin Exp Rheumatol* Online.

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