Genome-wide analysis of 5-hmC in the peripheral blood of systemic lupus erythematosus patients using an hMeDIP-chip

WEIGUO SUI^{1*}, QIUPEI TAN^{1*}, MING YANG¹, QIANG YAN¹, HUA LIN¹, MINGLIN OU¹, WEN XUE¹, JIEJING CHEN¹, TONGXIANG ZOU¹, HUANYUN JING¹, LI GUO¹, CUIHUI CAO¹, YUFENG SUN¹, ZHENZHEN CUI¹ and YONG DAI²

¹Guangxi Key Laboratory of Metabolic Diseases Research, Central Laboratory of Guilin 181st Hospital, Guilin, Guangxi 541002; ²Clinical Medical Research Center, the Second Clinical Medical College of Jinan University (Shenzhen People's Hospital), Shenzhen, Guangdong 518020, P.R. China

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Abstract. Systemic lupus erythematosus (SLE) is a chronic, potentially fatal systemic autoimmune disease characterized by the production of autoantibodies against a wide range of self-antigens. To investigate the role of the 5-hmC DNA modification with regard to the onset of SLE, we compared the levels 5-hmC between SLE patients and normal controls. Whole blood was obtained from patients, and genomic DNA was extracted. Using the hMeDIP-chip analysis and validation by quantitative RT-PCR (RT-qPCR), we identified the differentially hydroxymethylated regions that are associated with SLE. There were 1,701 genes with significantly different 5-hmC levels at the promoter region in the SLE patients compared with the normal controls. The CpG islands of 3,826 genes showed significantly different 5-hmC levels in the SLE patients compared with the normal controls. Out of the differentially hydroxymethylated genes, three were selected for validation, including TREX1, CDKN1A and CDKN1B. The hydroxymethylation levels of the three genes were confirmed by RT-qPCR. The results suggested that there were significant alterations of 5-hmC in SLE patients. Thus, these differentially hydroxymethylated genes may contribute to the pathogenesis of SLE. These findings show the significance of 5-hmC as a potential biomarker or promising target for epigenetic-based SLE therapies.

*Contributed equally

Introduction

Systemic lupus erythematosus (SLE) is a typical systemic autoimmune disease, involving diffuse connective tissues (1) and is characterized by immune inflammation. SLE has a complex pathogenesis (2), involving genetic, immunologic and environmental factors. Thus, it may result in damage to multiple tissues and organs, especially the kidneys (3). SLE arises from a combination of heritable and environmental influences.

Epigenetics, the study of changes in gene expression that occur without changes in the DNA sequence, have been suggested to underlie age-related dysfunction and associated disorders (5). The major epigenetic mechanisms include DNA methylation, histone modifications and microRNAs. Recent findings (4) have shown that epigenetic abnormalities are closely correlated with the pathogenesis of SLE. Epigenetic studies may provide clues to elucidate the pathogenesis of SLE and develop new strategies to treat this disease.

DNA hydroxymethylation (5-hydroxymethylcytosine, 5-hmC) (6,7) is a newly described epigenetic modification. It is an oxidative product of the well-known DNA methylation (5-methylcytosine, 5-mC) and catalyzed by the ten eleven translocation (TET) family of enzymes (8), a family of enzymes dependent on 2-oxoglutarate and Fe(II) *in vitro* and *in vivo*.

The methylation of cytosine-guanine dinucleotides (CpG) with C (9) is a common epigenetic modification in mammals and is also widespread in animals and plants. As an important epigenetic modification, 5-mC regulates genomic functions, such as gene transcription, X-chromosome inactivation, imprinting, genetic mutation and chromosome stability (10-12). 5-mC is only one component of a dynamic epigenetic regulatory network of DNA modifications that also includes 5-hmC, 5-formylcytosine and 5-carboxylcytosine. The reversible methylation of N6-methyladenosine in RNA has also been demonstrated (13).

5-hmC was first found in bacteriophage DNA in 1952. It was utilized several decades ago, only after its recent identification in DNA from murine brain and stem cells rendered 5-hmC a major focus of epigenomic investigations (14). The lower affinity of methyl-binding proteins to 5-hmC compared with 5-mC suggests that this modification may have a distinct

Correspondence to: Professor Yong Dai, Clinical Medical Research Center, the Second Clinical Medical College of Jinan University (Shenzhen People's Hospital), No. 1017 Dongmen North Road, Shenzhen, Guangdong 518020, P.R. China E-mail: daiyong2222@gmail.com

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role in gene expression regulation. However, 5-hmC is also involved in the DNA demethylation process (15,16).

To obtain a deeper understanding of the role of 5-hmC with regard to the onset of SLE, we generated genome-wide maps of 5-hmC in patients with SLE and healthy controls by performing hydroxymethyl-DNA immunoprecipitation followed by massively parallel sequencing with an Illumina Genome Analyzer (hMeDIP-chip).

Materials and methods

Patients and controls. Whole blood samples from 15 SLE patients and 15 normal controls were obtained from the 181st Hospital of Guilin (China), between January and September, 2011. The SLE diagnoses were confirmed based on pathology and clinical evidence following the American Rheumatism Association classification criteria (1987).

Written informed consent was obtained from all the subjects or their guardians. The use of biopsy material for studies beyond routine diagnosis was approved by the local ethics committee. This study abides by the Helsinki Declaration on ethical principles for medical research involving human subjects.

Genomic DNA extraction and fragmentation. Blood samples were obtained from SLE patients (n=15, 5 μ l per subject pooled into one blood sample) and normal controls (n=15, 5 μ l per subject pooled into one blood sample). Genomic DNA (gDNA) was extracted from the SLE patients and normal control blood samples using a DNeasy Blood & Tissue kit (Qiagen, Fremont, CA, USA). The purified gDNA was then quantified and its quality assessed using a Nanodrop ND-1000 (Table I). The genomic DNA from each sample pool was sonicated to ~200-1000 bp using a Bioruptor sonicator (Diagenode, Denville, NJ, USA) on the 'Low' setting for 10 cycles of 30 sec 'ON' and 30 sec 'OFF'. The gDNA and each sheared DNA sample were analyzed on an agarose gel.

GO analysis of differentially expressed 5-hmC. To investigate the specific functions of the differentially expressed 5-hmC in the developmental process of SLE, the 5-hmC targets of each differentially expressed 5-hmC were identified by GO categories. The GO categories are derived from gene ontology, which comprise three structured networks of defined terms that describe gene product attributes.

Pathway analysis of differentially expressed 5-hmC. Pathway analysis is a functional analysis mapping genes to KEGG pathways. To evaluate the effect of SNP-to-gene mapping strategy on pathway analysis, we also mapped SNPs to genes within differentially expressed 5-hmC.

Immunoprecipitation. One microgram of the sonicated genomic DNA was used for immunoprecipitation using a mouse monoclonal anti-5-hydroxymethylcytosine antibody (Diagenode). Prior to immunoprecipitation, the spike-in control sequences were mixed with the genomic DNA fragments. The DNA was then heat-denatured at 94°C for 10 min, rapidly cooled on ice, and immunoprecipitated with 1 μ l of primary antibody overnight at 4°C with rocking agitation in 400 μ l of immunoprecipitation buffer (0.5% BSA in PBS).

To recover the immunoprecipitated DNA fragments, 200 μ l of anti-mouse IgG magnetic beads were added and incubated for an additional 2 h at 4°C with agitation. After immunoprecipitation, a total of five immunoprecipitation washes were performed with ice-cold immunoprecipitation buffer. The washed beads were resuspended in TE buffer with 0.25% SDS and 0.25 mg/ml proteinase K for 2 h at 65°C and then allowed to cool to room temperature. The hMeDIP DNA fragments were purified using Qiagen MinElute columns (Qiagen).

DNA labeling and array hybridization. For DNA labeling, the NimbleGen Dual-Color DNA Labeling kit was used according to the manufacturer's instructions as detailed in the NimbleGen hMeDIP-chip protocol (NimbleGen Systems, Inc., Madison, WI, USA). DNA (1 μ g) from each sample was incubated for 10 min at 98°C with 1 OD of Cy5-9mer primer (IP sample) or Cy3-9mer primer (Input sample). Then, 100 pmol of deoxynucleoside triphosphates and 100 units of the Klenow fragment (New England Biolabs, Beverly, MA, USA) were added, and the mixture was incubated at 37°C for 2 h. The reaction was stopped by adding 0.1X volume of 0.5 MEDTA, and the labeled DNA was purified by isopropanol/ethanol precipitation. The microarrays were hybridized at 42°C for 16-20 h with Cy3/5-labeled DNA in Nimblegen hybridization buffer/ hybridization component A in a hybridization chamber (Hybridization System - Nimblegen Systems, Inc.). Following hybridization, washing was performed using the Nimblegen Wash Buffer kit (Nimblegen Systems, Inc.). For array hybridization, Roche NimbleGen's Promoter plus CpG Island Array was used, which is a 385K array containing 28,226 CpG islands and well-characterized promoter regions (approximately -800 to +200 bp relative to the TSSs) that were completely covered by ~385,000 probes.

Quantitative RT-PCR verification of 5-hmC. The DNA was reverse transcribed to cDNA using gene-specific primers (Table II). The cycle parameters for the PCR reactions were 95°C for 10 min followed by 40 cycles of a denaturing step at 95°C for 10 sec and an annealing/extension step at 60°C for 60 sec. The relative amount of each gene was described using the equation 2- Δ Ct, where Δ Ct = (CtmRNA-CtU6). The genes analyzed included *TREX1*, *CDKN1A* and *CDKN1B*.

Results

hMeDIP-chip. Using specific antibodies, we performed hMeDIP-chip (17) on two samples: SLE patients and normal controls. To determine the 5-hmC status of a comprehensive set of human promoters, we enriched the DNA from whole blood samples for hydroxymethylated DNA using hMeDIP-chip methodology combined with microarray detection. The selected platform was a single array design that included 28,226 CpG islands and all the Ref gene promoter regions (approximately -800 to +200 bp relative to the TSSs) that were completely covered by ~385,000 probes. The median probe spacing was 101 bp.

DMR analysis using the MEDME method. To accurately quantify the CpG 5-hmC levels, we used a new analytical methodology, MEDME (modeling experimental data with

Sample ID	OD260/2 80 ratio	OD260/2 30 ratio	Conc. (ng/µl)	Volume (µl)	Total amount (ng)
Control	1.81	2.05	41.60	100	4160.00
SLE	1.63	1.89	32.38	100	3238.00

Table I. DNA quantification and quality assurance by NanoDrop spectrophotometer.

For spectrophotometer, the optical density (OD) A260/A280 ratio was required to be close to 1.8 for pure DNA (ratios between 1.7 and 2.0 were acceptable). The OD A260/A230 ratio was required to be >1.8.

Table II. Reverse-transcription and RT-qPCR primers.

Gene name	RT-qPCR primers	Annealing temperature (°C)	Product length (bp)
TREX1	F: 5'-GTGTTCCAAGTGCTGCCAAA-3' R: 5'-CATAAAGAGCGTGGGCTACATAC-3'	60	245
CDKN1A	F: 5'-AGCCTTCCTCACATCCTCCTT-3' R: 5'-GACGGCCAGAAAGCCAATC-3'	60	207
CDKN1B	F: 5'-GCCAGCCAGAGCAGGTTT-3' R: 5'-GATTGACACGGCGAGTCTATTT-3'	60	224
F, forward; R, rever	rse.		

hMeDIP enrichment), to improve the evaluation and interpretation of the hMeDIP-derived 5-hmC estimates. MEDME utilizes the absolute 5-hmC score (AHS) as the value for DNA hydroxymethylation, which is calculated based on the weighted count of the hydroxymethylated CpG dinucleotides in a 1 kb window centered at each probe. The AHS has been verified to be a more accurate and sensitive measurement of 5-hmC levels than the log-ratio. The MEDME method also provides a relative 5-hmC score (RHS) that normalizes the AHS to the total number of CpGs represented by CpGw. This method allows investigators to obtain a relative measurement of the 5-hmC that is independent of the CpG density of the corresponding region. The RMS is especially useful when comparing regions with different CpG densities.

Promoter classes in relation to CpG frequency. Approximately 70% of human genes are associated with promoter CpG islands, whereas the remaining promoters tend to be depleted in CpGs. The presence of 5-hmC in promoter regions is associated with high levels of transcription, which is consistent with a role for 5-hmC in the maintenance and promotion of gene expression. This effect is also partially dependent on the CpG density of the promoter. Based on the CpG density, the CpG ratio and length of the CpG-rich region, the promoters are subdivided into three classes: high (HCP), low (LCP), and intermediate (ICP) CpG density.

These classes are defined as follows: i) High-CpG-density promoters (HCP) are promoters containing a 500-bp interval within the region from 0.7 kb upstream to 0.2 kb downstream of the TSS with a GC percentage \geq 55% and a CpG observed-to-expected ratio (O/E) \geq 0.6. ii) Low-CpG-density promoters



Figure 1. The definition of the relationship CPG and the transcription region.

(LCP) are promoters containing no 500 bp interval with a CpG O/E \geq 0.4. iii) Intermediate-CpG-density promoters (ICP) include the remaining promoters that were not classified as HCP or LCP.

CpG island 5-hmC. Mammalian genomes are punctuated by DNA sequences that contain an atypically high frequency of CpG sites termed CpG islands (CGIs). These sequences are characterized as \geq 200 bp in length with a GC content of 50% and a CpG O/E of 0.6.

CpG islands can be grouped into three classes based on their distance to RefSeq annotated genes: i) Promoter islands occur from approximately -10 to +0.5 kb around the transcription start site. ii) Intragenic islands occur from 0.5 kb downstream of the transcription start site to the site of transcription termination. iii) Intergenic islands include all other CpG islands that were not classified as being in the promoter or intragenic category (Fig. 1).



Figure 2. DNA 385K promoter of hydroxymethylated and unhydroxymethylated promoters compared between the SLE patients and the normal controls. (A) Classification of all, hydroxymethylated, or unhydroxymethylated promoters with high (HCP), intermediate (ICP), and low (LCP) CpG content. (B) Breakdown of hydroxymethylation status for HCP, ICP, and LCP promoters. (C) Percentage of genes with hydroxymethylated promoters in CpG islands.

Genome-wide profiling of promoter DNA 5-hmC. Based on the data obtained, we examined the CpG content in the pool of hydroxymethylated promoters compared to non-hydroxymethylated promoters that exhibited significant differences in 5-hmC levels between the SLE patients and normal controls. We found that 65.95% of hydroxymethylated genes belonged to the HCP cluster, which is similar to the average occurrence of HCP genes genome-wide (67.82%) (Fig. 2A). Similarly, 69.85% of the non-hydroxymethylated genes were associated with HCPs (Fig. 2A). A detailed analysis of the distribution of the hydroxymethylated probes over these promoters, which contained at least one CpG island by definition, indicated that 75.21% of the HCP genes had a hydroxymethylated probe that overlapped with the CpG island itself (Figs. 1 and 2C). By contrast, ~45% of the ICP and LCP genes were characterized as hydroxymethylated genes (Fig. 2B). We conclude that DNA hydroxymethylation in the blood of SLE patients primarily occurs at HCP promoters or at nonpromoter-CpG islands within HCP genes.

GO analysis of differentially expressed 5-hmC. To investigate the specific functions of the differentially expressed 5-hmC in the developmental process of SLE, the 5-hmC targets of each differentially expressed 5-hmC were identified by GO categories. The GO categories were derived from gene ontology, comprising three structured networks of defined terms that describe gene product attributes. The P-value denotes the significance of GO term enrichment in the differentially expressed 5-hmC list. Thus, the lower the P-value, the more significant the GO term, with P<0.05 being recommended. In terms of the GO database, the differentially expressed proteins encoded by these genes were divided into three categories: biological process, cell component and molecular function (Fig. 3). Through GO analysis for differentially expressed 5-hmC genes, we found that 71 differentially expressed 5-hmC genes with annotation terms being linked to the GO biological process categories, 30 being linked to the cell component and 20 being linked to the molecular function, with P<0.01. Details of the cell component categories, molecular function ontology, biological process ontology are presented in Table III.

Pathway analysis of differentially expressed 5-hmC. Pathway analysis is a functional analysis mapping genes to KEGG pathways. The P-value (EASE-score, Fisher P-value or Hypergeometric P-value) denotes the significance of the pathway correlated with the following conditions: the lower the P-value, the more significant the pathway, with P=0.05 as the cut-off value. In order to evaluate the influence of SNP-togene mapping strategy on the pathway analysis, we mapped SNPs to genes within differentially expressed 5-hmC.

In terms of the Pathway database, 17 pathways were significant (P<0.05). Differentially expressed 5-hmC is shown in Fig. 4, while details of the pathways are present in Table IV. Furthermore, the *CDKN1A* and *CDKN1B* genes contributed to the ErbB (P=0.01073062), P13-Akt (P=0.04341327), and HIF-1 (P=0.04345306) signaling pathways.

Comparison of 5-hmC status between SLE patients and normal controls. By applying the analysis procedure described



Figure 3. (A) Signal GO term of differentially expressed genes in biological process. (B) Signal GO term of differentially expressed genes in cellular component. (C) Signal GO term of differentially expressed genes in molecular function.

above to the sequencing results, we found that 1,701 gene promoter regions showed significantly different levels of 5-hmC in the SLE patients compared with the normal controls. Of these genes, 884 exhibited increased 5-hmC and 817 exhibited decreased 5-hmC (Fig. 5A). The CpG islands of 3,826 genes showed significant differences in 5-hmC levels in

GO:0008328

GO:0031974

Gene Enrichment GO ID Term (n) P-value score Molecular function 975 GO:0005488 8.47676E-06 5.071770331 Binding GO:0005515 Protein binding 574 0.0006979 3.156206958 GO:0008601 Protein phosphatase type 2A regulator activity 6 0.002390223 2.621561587 Amine transmembrane transporter activity 14 GO:0005275 0.002741565 2.562001469 GO:0046943 Carboxylic acid transmembrane transporter activity 16 0.003032561 2.518190484 5 GO:0008198 Ferrous iron binding 2.515906422 0.003048552 GO:0031267 Small GTPase binding 19 0.003112164 2.506937561 GO:0050662 Coenzyme binding 26 0.003164007 2.499762629 GO:0005342 Organic acid transmembrane transporter activity 16 0.003738627 2.427287911 GO:0019903 Protein phosphatase binding 12 0.003795492 2.420731959 GO:0004683 Calmodulin-dependent protein kinase activity 6 0.004182328 2.37858187 5 0.004284399 GO:0015248 Sterol transporter activity 2.368110104 Solute:sodium symporter activity 10 GO:0015370 0.005096459 2.292731449 Symporter activity 19 GO:0015293 0.005283676 2.277063859 GO:0003677 DNA binding 210 0.00545348 2.263326261 GO:0017016 Ras GTPase binding 17 0.005511619 2.258720783 290 GO:0003676 Nucleic acid binding 0.005672365 2.246235825 GO:0036094 Small molecule binding 229 0.00603527 2.219303294 GO:0000166 Nucleotide binding 215 0.006277361 2.202222878 GO:0019902 Phosphatase binding 15 0.008158707 2.088378687 Cellular component 991 GO:0044424 Intracellular component 1.25802E-06 5.900311838 1011 GO:0005622 Intracellular 1.96706E-06 5.706182597 GO:0005737 Cytoplasm 751 1.1809E-05 4.927788392 Organelle GO:0043226 863 1.23569E-05 4.908089407 GO:0043229 Intracellular organelle 860 2.02157E-05 4.694310928 GO:0044444 Cytoplasmic component 556 0.000162517 3.789099975 GO:0043227 Membrane-bound organelle 773 0.000201161 3.696456379 GO:0043231 Intracellular membrane-bound organelle 771 0.000263573 3.579099179 GO:0005634 Nucleus 503 0.000511564 3.291100377 GO:0044422 Organelle component 518 0.000698363 3.155918681 GO:0044446 Intracellular organelle component 511 0.000826581 3.082714383 GO:0005815 Microtubule organizing center 54 0.001500698 2.823706722 GO:0044464 Cell component 1146 2.79607593 0.001599278 1146 GO:0005623 Cell 0.00163091 2.78757007 GO:0015630 Microtubule cytoskeleton 86 0.001672538 2.776623893 85 GO:0031988 Membrane-bound vesicle 0.002151442 2.667270359 GO:0000159 Protein phosphatase type 2A complex 6 0.002383019 2.622872453 163 GO:0005856 Cytoskeleton 0.002653136 2.576240518 5 GO:0030312 External encapsulating structure 0.003040318 2.517080953 GO:0016023 Cytoplasmic membrane-bound vesicle 82 0.003540727 2.450907507 85 Cytoplasmic vesicle GO:0031410 0.005938359 2.226333534 215 GO:0044428 Nuclear component 0.006110243 2.213941539 Non-membrane-bound organelle GO:0043228 265 0.006188271 2.208430644 265 GO:0043232 Intracellular non-membrane-bound organelle 0.006188271 2.208430644 Vesicle 88 GO:0031982 0.006466493 2.189331194 37 GO:0005874 Microtubule 0.007122082 2.147393031 4 0.007554527 GO:0043240 Fanconi anaemia nuclear complex 2.12179274 GO:0005829 201 0.007559368 Cytosol 2.121514538

0.008472321

0.009528722

6

228

2.071997622

2.020965349

Ionotropic glutamate receptor complex

Membrane-enclosed lumen

Table III. Functional analysis of genetic differences of 5-hmC (P<0.01).

Table III. Continued.

GO ID	Term	Gene (n)	P-value	Enrichment score
		()		
GO:0009987	Cell process	1046	4 20173E-07	6 376571542
GO:00055507	DNA metabolic process	93	0.000371782	3 429711695
GO:000235	Neurotransmitter hiosynthetic process	6	0.000371782	3 392331799
GO:0042130 GO:0051179	Localization	374	0.000503121	3 2983276
GO:0033554	Cell response to stress	114	0.000534186	3 272307489
GO:0016043	Cell component organization	344	0.000617972	3 209030892
GO:0008219	Cell death	166	0.000636118	3 19646263
GO:0006215	Death	166	0.000672212	3 172493732
GO:0071840	Cell component organization or biogenesis	352	0.000808943	3 092082017
GO:0071705	Nitrogen compound transport	29	0.000836139	3.077721402
GO:0010950	Positive regulation of endopentidase activity	18	0.000934594	3 029376965
GO:0012501	Programmed cell death	152	0.000944228	3 024923241
GO:0071702	Organic substance transport	59	0.001039557	2.983151486
GO:0021987	Ccerebral cortex development	12	0.001156639	2.9368022
GO:0006915	Apoptotic process	150	0.001275381	2.894360003
GO:0015697	Quaternary ammonium group transport	6	0.001359721	2.866550106
GO:0006308	DNA catabolic process	13	0.001439981	2.841643257
GO:0010952	Positive regulation of peptidase activity	18	0.001648322	2.782957883
GO:0030301	Cholesterol transport	12	0.001863628	2.729640865
GO:0015918	Sterol transport	12	0.002166069	2.664327795
GO:0021543	Pallium development	15	0.002411739	2.617669728
GO:0042632	Cholesterol homeostasis	11	0.00280871	2 551493105
GO:0055092	Sterol homeostasis	11	0.00280871	2.551493105
GO:0007169	Transmembrane receptor protein tyrosine kinase	66	0.003092099	2.509746586
00.000/10/	signaling pathway	00	0.000002000	2.307710300
GO:0006281	DNA repair	45	0.00309275	2.509655117
GO:0006919	Activation of cysteine-type endopeptidase	14	0.003187141	2.496598699
	activity involved in apoptotic process			
GO:0097202	Activation of cysteine-type endopeptidase activity	14	0.003187141	2.496598699
GO:0007049	Cell cycle	131	0.003246836	2.488539594
GO:0006950	Response to stress	254	0.003252788	2.487744307
GO:0060317	Cardiac epithelial to mesenchymal transition	5	0.003278753	2.484291311
GO:0015837	Amine transport	24	0.003396431	2.468977217
GO:0010033	Response to organic substance	156	0.003703461	2.431392194
GO:0043280	Positive regulation of cysteine-type endopeptidase	16	0.003962	2.402085511
	activity involved in apoptotic process			
GO:2001056	Positive regulation of cysteine-type endopeptidase activity	16	0.003962	2.402085511
GO:0010941	Regulation of cell death	119	0.004400697	2.35647858
GO:0034641	Cellular nitrogen compound metabolic process	465	0.004692655	2.328581332
GO:0008629	Induction of apoptosis by intracellular signals	15	0.004728501	2.32527652
GO:0071842	Cellular component organization at the cellular level	268	0.0048397	2.315181593
GO:0032677	Regulation of interleukin-8 production	8	0.004962569	2.304293443
GO:0006807	Nitrogen compound metabolic process	473	0.005208958	2.283249159
GO:0042981	Regulation of apoptotic process	115	0.005273137	2.277930908
GO:0071294	Cell response to zinc ion	4	0.005446325	2.26389645
GO:0006139	Nucleobase-containing compound metabolic process	431	0.005604946	2.251428588
GO:0006810	Transport	302	0.005935568	2.22653772
GO:0071841	Cell component organization or biogenesis at the cellular level	275	0.00611302	2.213744154
GO:0051716	Cell response to stimulus	413	0.006239288	2.204864975
GO:0032365	Intracellular lipid transport	5	0.006266841	2.20295135

Table III. Continued.

		Gene		Enrichment
GO ID	Term	(n)	P-value	score
GO:0040007	Growth	76	0.006279845	2.20205109
GO:0044272	Sulfur compound biosynthetic process	11	0.006528232	2.185204402
GO:0043067	Regulation of programmed cell death	115	0.006686996	2.174768905
GO:0007167	Enzyme-linked receptor protein signaling pathway	83	0.006693713	2.174332938
GO:0030168	Platelet activation	28	0.007338694	2.134381237
GO:0015695	Organic cation transport	7	0.007484604	2.125831149
GO:0014066	Regulation of phosphatidylinositol 3-kinase cascade	9	0.007501308	2.124863025
GO:0050794	Regulation of the cell process	631	0.007609603	2.118638012
GO:0044260	Cellular macromolecule metabolic process	530	0.007722583	2.11223743
GO:0042157	Lipoprotein metabolic process	15	0.00781696	2.10696213
GO:0051234	Establishment of localization	305	0.007999858	2.096917735
GO:0003203	Endocardial cushion morphogenesis	4	0.008030603	2.095251834
GO:0042149	Cell response to glucose starvation	4	0.008030603	2.095251834
GO:0045540	Regulation of cholesterol biosynthetic process	4	0.008030603	2.095251834
GO:0043065	Positive regulation of apoptotic process	61	0.008093257	2.091876684
GO:0035556	Intracellular signal transduction	173	0.008130715	2.089871248
GO:0032637	Interleukin-8 production	8	0.008361973	2.077691232
GO:0090304	Nucleic acid metabolic process	365	0.00864538	2.063215898
GO:0046686	Response to cadmium ion	7	0.009025274	2.044539606
GO:0006586	Indolalkylamine metabolic process	6	0.009194049	2.036493163
GO:0009225	Nucleotide-sugar metabolic process	6	0.009194049	2.036493163
GO:0042430	Indole-containing compound metabolic process	6	0.009194049	2.036493163
GO:0006974	Response to DNA damage stimulus	61	0.009681709	2.014047956
GO:0043068	Positive regulation of programmed cell death	61	0.009681709	2.014047956

GO ID, the ID of gene ontology terms used in the Gene Ontology Project; Term, the name of the gene ontology term; Enrichment score, the enrichment score value of GO ID, which equals [-log10 (P-value)]; P-value, the significance testing value of the GOID, result from the topGO of bioconductor. If the P-value is lower than 1e-299, the P-value will be adjusted as the value of 1e-299.



Figure 4. Pathway barplot explanation of differentially expressed genes.

the SLE patients compared with the normal controls. Of these genes, 2,034 exhibited increased 5-hmC and 1,792 exhibited decreased 5-hmC (Fig. 5B).

Pie chart A shows the chromosomal locations of the 884 genes that were hyper-hydroxymethylated within the promoter

region in the SLE patients compared with the normal controls (clockwise from chromosome 1 to the X and Y sex chromosomes). The percentage of genes hyper-hydroxymethylated on chromosome 1 was 10% (Fig. 6A). Pie chart B shows the chromosomal locations of the 2,034 genes that were

Table IV. Transduction pathway analysis in	in 5-hmC differences gene	s.
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KEGG Pathways	Fisher (n)	Gene P-value	Enrichment score	Genes
Histidine metabolism - Homo sapiens	0.000632576	8	3.198888	ACY3, ALDH1B1, ALDH3A2, ALDH3B1, HAL, HEMK1, MAOA, WBSCR22
Circadian entrainment - Homo sapiens	0.001569437	16	2.804256	ADCY4, ADCYAP1R1, CACNA1G, CAMK2D, GNAS, GNG13, GRIA1, GRIN2A, GRIN2C, GRIN2D, KCNJ5, MAPK1, NOS1, PER1, PRKG1, RYR2
Glycerolipid metabolism - Homo sapiens	0.005711304	10	2.243265	AGPAT3, ALDH1B1, ALDH3A2, CEL, DGKH, DGKQ, DGKZ, GPAM, LIPG, PPAP2A
Calcium signaling pathway - <i>Homo sapiens</i>	0.005990646	23	2.222526	ADCY4, ADRA1D, ADRB2, ATP2B3, CACNA1G, CAMK2D, CAMK4, CD38, CYSL-TR1, EGFR, GNAS, GRIN2A, GRIN2C, GRIN2D, HTR7, MYLK2, MYLK3, NOS1, P2RX1, PDE1B, PHKG2, PLCD4, RYR2
Amyotrophic lateral sclerosis (ALS) - Homo sapiens	0.0104193	9	1.982162	APAF1, CYCS, DERL1, GRIA1, GRIN2A, GRIN2C, GRIN2D, NEFH, NOS1
ErbB signaling pathway - Homo sapiens	0.01073062	13	1.969375	CAMK2D, CDKN1A, CDKN1B, CRK, CRKL, EGFR, ELK1, MAPK1, NCK1, NRG2, NRG3, PIK3R3, PTK2
Glycine, serine and threonine metabolism - <i>Homo sapiens</i>	0.01823201	7	1.739166	BHMT, CTH, DAO, GNMT, MAOA, PSPH, SDSL
Amphetamine addiction - Homo sapiens	0.02439724	10	1.612659	CAMK2D, CAMK4, CREB3L2, GNAS, GRIA1, GRIN2A, GRIN2C, GRIN2D, MAOA, TH
Cocaine addiction - Homo sapiens	0.02669436	8	1.57358	BDNF, CREB3L2, GNAS, GRIN2A, GRIN2C, GRIN2D, MAOA, TH
Lysosome - Homo sapiens	0.03098124	15	1.508901	ABCA2, AP3M1, AP3S2, AP4B1, ARSA, ATP6V0B, ATP6V1H, CLTA, CTNS, CTSL2, HEXB, IGF2R, NPC1, NPC2, SLC17-A5
Glycerophospholipid metabolism - <i>Homo sapiens</i>	0.03160531	12	1.50024	AGPAT3, CHAT, DGKH, DGKQ, DGKZ, ETNK1, GNPAT, GPAM, LYPLA1, PEMT, PPAP2A, TAZ
Fanconi anemia pathway - Homo sapiens	0.03646938	8	1.438072	BLM, FANCB, FANCD2, FANCE, FANCF, RPA2, STRA13, WDR48
Alcoholism - <i>Homo sapiens</i>	0.03755879	20	1.425288	BDNF, CAMK4, CREB3L2, GNAS, GNG13, GRIN2A, GRIN2C, GRIN2D, H2AFV, H2BFM, HIST1H3F, HIST1H4D, HIST2H2AB, HIST2H2BF, HIST2H3D, HIST3H3, MAOA, MAPK1, NTRK2, TH
PI3K-Akt signaling pathway - Homo sapiens	0.04341327	34	1.362377	BCL2L11, CDKN1A, CDKN1B, CHAD, COL6A1, CREB3L2, EGFR, EPOR, FASLG, FGF1, FLT1, GNG13, HSP90AA1, HSP90B1, INSR, ITGAV, ITGB4, JAK2, KITLG, LAMA5, LAMC3, MAPK1, PIK3R3, PPP2R1A, PPP2R2B, PPP2R3A, PPP2R5E, PRKCZ, PTEN, PTK2, SGK1, THEM4, TLR2, YWHAZ

Table IV. Continued.

KEGG pathways	Fisher (n)	Gene P-value	Enrichment score	Genes
HIF-1 signaling pathway - Homo sapiens	0.04345306	13	1.36198	ALDOA, CAMK2D, CDKN1A, CDKN1B, EGFR, FLT1, IFNGR1, INSR, MAPK1, PGK1, PIK3R3, SLC2A1, TF
DNA replication - Homo sapiens	0.04358268	6	1.360686	MCM2, PCNA, POLA2, RFC2, RNASEH2C, RPA2
Focal adhesion - <i>Homo sapiens</i>	0.04412979	22	1.355268	BCAR1, CHAD, COL6A1, CRK, CRKL, EGFR, ELK1, FLNC, FLT1, ITGAV, ITGB4, LAMA5, LAMC3, MAPK1, MYL12A, MYLK2, MYLK3, PIK3R3, PPP1R12B, PTEN, PTK2, VCL

Fisher P-value, the enrichment P-value of the Pathway ID using Fisher's exact test; Enrichment score: the enrichment score value of the pathway ID, which equals [-log10 (P-value)].



Figure 5. Different DNA hydroxymethylation level in the blood of SLE patients compared with the normal controls. (A) Different DNA hydroxymethylation levels in gene promoter (-800 to +200 bp), in the whole blood cells of SLE patients compared with the normal controls. (B) Different DNA hydroxymethylation levels in CpG Islands, in the whole blood cells of SLE patients compared with the normal controls.

hyper-hydroxymethylated within the CpG islands in the SLE patients compared with the normal controls (clockwise from chromosome 1 to the X and Y sex chromosomes). The percentage of genes hyper-hydroxymethylated on chromosome 29 was 9% (Fig. 6B).

The 5-hmC modifications of 15 selected genes are shown in Table V. The selected genes showed the greatest differences. Of these genes, we selected three prime repair exonuclease 1 (TREX1), cyclin-dependent kinase inhibitor 1A (p21, Cip1; CDKN1A), and cyclin-dependent kinase inhibitor 1B (p27, Kip1; CDKN1B) for verification. The microarray data were consistent with the RT-qPCR results (Table VI) showing that *TREX1*, *CDKN1A*, and *CDKN1B* exhibited significantly increased levels of 5-hmC. The three genes showed the largest differences in 5-hmC levels and may therefore be associated with SLE.

Discussion

The 5-hmC modification has been identified in mammalian DNA (6), but its broader role in epigenetics remains to be resolved. Early evidence suggests a few putative mechanisms that have potentially important implications (18): i) Conversion of methylcytosine (5-mC) to 5-hmC may displace methyl-binding proteins (MBPs). MeCP2, for instance, does not bind to 5-hmC. ii) 5-hmC may induce demethylation by interfering with the methylation maintenance function of DNMT1 during cell division. iii) 5-hmC may have its own specific binding proteins that alter the chromatin structure or DNA methylation patterns.

5-hmC was previously observed; however, little is known regarding its subtle interrelationship with other epigenetic modifications and potential functional significance in human



Figure 6. Pie charts showing the chromosomal location of the upregulated genes in SLE patients that compared to normal controls. (A) Pie charts showing the chromosomal location of the 884 genes that were upregulated in the gene promoter (clockwise from chromosome 1 to X and Y sex chromosomes). The percentage of genes upregulated on chromosome 1 is 10%. (B) Pie charts showing the chromosomal location of the 2,034 genes that were upregulated in CpG islands (clockwise from chromosome 1 to X and Y sex chromosomes). The percentage of genes upregulated on the chromosome 1 is 9%.

Peak ID	Gene name	Peak region	Peak score	Peak M-value	
4636	RTN1	Chr14: 59263472-59264121	4.71	1.26471321387763	
3845	S100A7L2	Chr1: 151678931-151679801	4.55	0.967562504015608	
5554	ZNRF4	Chr19: 5406466-5407310	4.48	1.10626932760703	
5087	CPNE7	Chr16: 88168557-88169204	4.37	1.37675784921923	
7131	FZD3	Chr8: 28406910-28407744	4.35	1.00487584265558	
4370	LAYN	Chr11: 110916352-110917416	4.32	0.86128687613001	
6211	LZTR1	Chr22: 19665877-19666606	4.32	1.21336961342519	
3824	PRMT6	Chr1: 107400534-107401083	4.23	0.934008953032345	
7529	VMA21	ChrX: 150315981-150316530	4.13	1.16048247400229	
6560	FREM3	Chr4: 144840477-144841631	4.09	1.18891146363541	
3911	HIST3H3	Chr1: 226679229-226679963	4.08	1.33700123117401	
6204	GNB1L	Chr22: 18221904-18222633	4.07	0.77963334561276	
6355	TREX1	Chr3: 48482232-48484048	2.7	0.450419940777759	
6777	CDKN1A	Chr6: 36754464-36763087	2.66	1.58157470937086	
679	CDKN1B	Chr12: 12761568-12766572	2.03	0.6574848262603	

Table V. The 15 selected genes with hydroxymethylation alterations between SLE and normal controls, identified by hmeDIP-seq.

Peak score, the average $-\log 10$ (P-value) from probes within the peak. The scores reflect the probability of positive enrichment (cut off = 2). Peak M-value, the median log2-ratio from probes within the peak region. The score reflects the hydroxymethylation level of the region.

disease. In this study, we selected 5-hmC as the target, performed an investigation using hMeDIP-chip, and investigated the hypothesis that 5-hmC is associated with the pathogenesis of SLE. We mainly analyzed the levels of 5-hmC in SLE patients and normal controls. The identified candidate genes with significant differences in 5-hmC levels are shown in Table V. This list includes genes associated with immunity, cell signal transduction, protein transcription and synthesis, ion channels and transporters, and the extracellular matrix.

Of the identified candidate genes, we found that *TREX1* was hyper-hydroxymethylated in the SLE patients compared

with the normal controls. Three prime repair exonuclease 1 (*TREX1*) is located on chromosome 3p21.31 and is also known as *CRV*, *AGS1*, *DRN3* or *HERNS*. This gene encodes a nuclear protein with 3' exonuclease activity, which may play a role in DNA repair and serve a proofreading function for DNA polymerase. Mutations in this gene result in Aicardi-Goutieres syndrome, chilblain lupus, Cree encephalitis, and other diseases of the immune system. Alternative splicing of this gene results in multiple transcript variants.

TREX1 plays a key role in the HIV-1 infection process (19). This protein degrades excess HIV-1 DNA, thereby preventing

	Input-IP						
Sample	Input (Ct)	IP(Ct)	%	Input (Ct)	Neg (Ct)	%	IP/neg
TREX1							
SLE	26.628	29.827	2.178	26.628	NA	NA	-
Normal controls	25.648	37.884	0.004	25.648	NA	NA	-
CDKN1A							
SLE	23.375	28.664	0.512	23.375	NA	NA	-
Normal controls	22.38	NA	NA	25.648	22.38	NA	-
CDKN1B							
SLE	26.474	33.43	0.161	26.474	NA	NA	-
Normal controls	27.284	33.512	0.267	27.284	NA	NA	-

Table VI. RT-qPCR	verification results.
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% Input = $2(Ct^{Input}-Ct^{ChIP}) \times Fd \times 100\%$. Fd refers to input dilution factor: For example, when 100 μ l sonicated sample was used for hMeDIP and 20 μ l sonicated sample was used as Input, Fd =1/5.

recognition by innate immunity receptors and the type I interferon response. Rare mutations in the TREX1 gene, the major mammalian 3'-5' exonuclease, have been reported in sporadic SLE cases (20,21). Some of these mutations have also been identified in a rare pediatric neurological condition featuring an inflammatory encephalopathy known as Aicardi-Goutieres syndrome (AGS) (22). The mutations have also been identified in patients with several different human diseases (23), such as Aicardi-Goutieres syndrome 1, and account for all the mutations in retinal vasculopathy with cerebral leukodystrophy. These mutations include null alleles, frameshift mutations and non-synonymous changes in the catalytic domains and the C-terminal region. In AGS, most TREX1 mutations are autosomal recessive and reduce exonuclease activity of the enzyme, in particular a transition of arginine to histidine at position 114 (R114H). Pulliero et al described mutations of the TREX1 gene in Aicardi-Goutières syndrome 1 that increase the ability of T-lymphocytes to inhibit the growth of neoplastic neuronal cells and related angiogenesis (24).

In SLE, most of the mutations reported thus far are heterozygous and are located outside of the catalytic domain in the C-terminal region. The functional significance of these mutations is unknown. To examine the frequency of mutations in the *TREX1* gene and their relationship with SLE, Namjou *et al* (25) genotyped 40 SNPs in the *TREX1* genomic region, including previously reported rare SNPs and more common tag SNPs that capture most of the variation in this region. Those authors reported results indicating that *TREX1* is involved in the lupus pathogenesis and is most likely essential for the prevention of autoimmunity. Gene Ontology (GO) term analysis shows that *TREX1* is mainly associated with the cell process, cellular nitrogen compound metabolic process, cell response to stress, intracellular component, intracellular, binding, and protein binding.

We also observed that *CDKN1A* was significantly hyper-hydroxymethylated and *CDKN1B* was significantly hypo-hydroxymethylated in the SLE patients compared with the normal controls. The cyclin-dependent kinase inhibitor 1A (p21, Cip1; CDKN1A) gene is located on chromosome 6p21.2 and is also known as P21, CIP1, SDI1, WAF1, CAP20, CDKN1, MDA-6 or p21CIP1. This gene encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of the cyclin-CDK2 or -CDK4 complexes and thus functions as a regulator of G1 cell cycle progression. The expression of this gene is closely regulated by the tumor-suppressor protein p53 and mediates the p53-dependent G1 cell cycle arrest in response to a variety of stress stimuli. This protein can interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and plays a regulatory role in DNA replication and DNA damage repair. This protein was reported to be specifically cleaved by CASP3-like caspases, which leads to marked activation of CDK2 and may be instrumental in the execution of apoptosis following caspase activation. Multiple alternatively spliced variants have been identified for this gene.

The *CDKN1A* gene that encodes a cell cycle inhibitor, p21 (WAF1/CIP1), is located in a region associated with SLE susceptibility. Decreased cell levels of p21 are associated with SLE (26,27). Single-nucleotide polymorphisms (SNPs) within the promoter and the first intron of *CDKN1A* are associated with SLE susceptibility. The minor allele A at nucleotide 899 of *CDKN1A* is associated with increased susceptibility to SLE and lupus nephritis and decreased cell levels of p21.

The cyclin-dependent kinase inhibitor 1B (p27, Kip1; CDKN1B) encodes a cyclin-dependent kinase inhibitor, which shares a limited similarity with the CDK inhibitor CDKN1A/p21. The encoded protein binds to and prevents the activation of the cyclin E-CDK2 or cyclin D-CDK4 complexes and thus controls G1 cell cycle progression. The degradation of this protein, which is triggered by its CDK-dependent phosphorylation and subsequent ubiquitination by SCF complexes, is required for the cellular transition from quiescence to the proliferative state.

CDKN1B (28) may lead to defects in apoptosis or autophagy and thus increase exposure of nuclear autoantigens to the immune system, and its potential role in autoimmunity is

supported by numerous functional studies. *CDKN1B* encodes p27Kip1, a cyclin-dependent kinase (CDK) inhibitor, which plays a critical role in the inhibition of cell-cycle progression, especially in T lymphocytes. p27Kip1 is essential for the induction of tolerance, a process believed to be at the center of autoimmune diseases such as SLE, and upregulation of p27Kip1 was found to correlate with the induction of anergy *in vitro* and tolerance *in vivo*. p27Kip1 is also involved in dendritic cell apoptosis, and the potential roles of the identified susceptibility genes in SLE etiology are noted. GO term analysis indicates that *CDKN1A* and *CDKN1B* are strongly associated with the cell process, intracellular component, intracellular, binding, and protein binding.

In this study, we systematically evaluated the genomewide levels of 5-hmC in the DNA of SLE patients and gained insight into the connections between key genes and 5-hmC in the context of SLE. Our results indicate that 5-hmC is involved in the disease state and these novel candidate genes may become potential biomarkers or future therapeutic targets. Future investigations are needed to clarify the roles of the identified hydroxymethylated candidate genes in the pathogenesis of SLE.

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