

Integrated analyses of a major histocompatibility complex, methylation and transcribed ultra-conserved regions in systemic lupus erythematosus

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Abstract. Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease which affects different organs and systems that, has a complex genetic inheritance, and is affected by both epigenetic and environmental risk factors. Previous studies on SLE have lacked the statistical power and genetic resolution to fully determine the influence of major histocompatibility complex (MHC) on SLE. In this study, in order to determine this influence, a total of 15 patients with SLE and 15 healthy controls were enrolled. MHC region capture technology, hMedIP-chip, transcribed ultra-conserved region (T-UCR) microarray and bioinformatics analysis were utilized for both groups. The results revealed methylated CpG enrichment at 6 loci in the MHC segment of SLE. We found 4 single-nucleotide polymorphisms (SNPs) in the CpG promoter of human leukocyte antigen-B (*HLA-B*) and 2 SNPs in chr6:29521110-29521833. No significant GO term or KEGG pathway enrichment was noted for an immune-correlated process in the SLE patients for the corresponding CpG-methylated genes. In this study, T-UCR was not discovered in the MHC segment. The analysis of SNPs (rs1050683, rs12697943, rs17881210, rs1065378, rs17184255 and rs16895070) and gene expression in peripheral blood lymphocytes indicated that these SNPs were associated with the occurrence of SLE. Further studies are warranted to examine the roles of these SNPs in the pathogenesis of SLE. Integrative analysis technology provided a view of the molecular signaling pathways in SLE.

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that affects different organs and systems and has a complex genetic inheritance (1). SLE has a complex etiology and is affected by both genetic and environmental factors (2). The major histocompatibility complex (MHC) located on chromosome 6p21 is one of the key factors that contribute to the development of SLE (3). The human leukocyte antigen (HLA) has been shown to be associated with susceptibility to SLE. Genome-wide association studies have demonstrated that variants within the MHC region confer the greatest genetic risk of developing SLE in European and Chinese populations. However, the causal variants remain elusive due to the tight linkage disequilibrium across disease-associated MHC haplotypes, the highly polymorphic nature of several MHC genes, and the heterogeneity of SLE phenotypes. The loci include *HLA-DPBI*, *HLA-G* and *MSH5*, which are independent of each other and *HLA-DRBI* alleles. These data highlight the usefulness of mapping disease susceptibility loci using a transancestral approach, particularly in a region as complex as the MHC, and offer a springboard for further fine-mapping, resequencing and transcriptomic analysis (4). Certain studies have suggested an association between MHC class I and II (*HLA-A*29*, *HLA-B*51*, *HLA-DRBI*15* and *HLA-DQBI*06*) and susceptibility to SLE in the Saudi population (5). In African-American women, a single-nucleotide polymorphism (SNP) which is closely associated with SLE, rs9271366, was found near the *HLA-DRBI* gene (6).

Although genetic variations within the MHC are associated with the development of SLE, its role in the development of the clinical manifestations and autoantibody production has not been well defined. A meta-analysis of 4 independent European SLE case collections was previously performed in an effort to identify associations between SLE sub-phenotypes and MHC SNP genotypes, HLA alleles, and variant HLA amino acids. The results provided strong evidence for a multilevel risk model for *HLA-DRBI*03:01* in SLE, wherein the association with anti-Ro and anti-La antibody-positive SLE is much stronger than with SLE without these autoantibodies (7). Despite the research which has been performed to date, a complete picture of these

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correlations has not yet been painted, and further studies are still needed to shed light on the associations between SLE and its susceptibility genes. In addition, novel methods should be used to investigate this subject.

Using a predictive bioinformatics algorithm, in a previous study, Mantila Roosa *et al* created a linear model of gene expression and identified 44 transcription factor-binding motifs and 29 miRNA-binding sites that were predicted to regulate gene expression across a time course. In addition to known sites, novel transcription factor-binding motifs and several novel miRNA-binding sites were identified throughout the time course. These time-dependent regulatory mechanisms may be important for controlling the loading-induced bone formation process (8). This integrated bioinformatics analysis method was also used in this study. Although the link between MHC and SLE has been proven, further investigations are likely to reveal the involvement of MHC in simple and complex genetic diseases, such as SLE. Indeed, we are interested in studying MHC, CpG methylation and transcribed ultra-conserved region (T-UCR) as a first step toward a better understanding of the regulation of gene expression in SLE. In the present study, we provide an extensive view of SLE based on an integrated bioinformatics analysis of MHC, CpG methylation and T-UCR datasets.

Materials and methods

Patients and controls. Whole blood samples from 15 patients with SLE (8 females; 7 males; aged 18-50 years, with an average age of 35.64 ± 11.27 years) and 15 normal healthy controls (8 females; 7 males; aged 20-45 years, with an average age of 33.47 ± 9.61 years) were collected from the 181st Hospital of Guilin, China, between January and September 2011. The SLE diagnoses were confirmed based on pathological and clinical evidence according to the American Rheumatism Association classification criteria (9,10). 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 Ethics Committee of the 181st Hospital of Guilin. This study abides by the Helsinki Declaration on Ethical Principles for Medical Research Involving Human Subjects.

MHC gene capture, hMeDIP-chip and T-UCR microarray analysis. Genomic DNA was isolated from peripheral blood samples. According to the MHC genomic sequence, a completely complementary probe was designed and fixed on a support and then applied to the genomic DNA after coupling with a probe connector. The unhybridized probe was washed away, and the probe that had hybridized with the DNA was eluted to directly build a library for DNA sequencing (HiSeq 2000 high-throughput sequencing). The MHC region capture technology was based on the NimbleGen SeqCap EZ Choice Library, enabling the deep sequencing coverage of the human MHC region. The data were analyzed using the Chi-squared test with Yates' correction for continuity.

Genomic DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen, Fremont, CA, USA). The sonicated genomic DNA (1 μ g) was used for immunoprecipitation with a mouse monoclonal antibody. For DNA labeling, a NimbleGen Dual-Color DNA Labeling kit was used according to the manufacturer's instructions detailed in the NimbleGen hMeDIP-chip

protocol (NimbleGen Systems, Inc., Madison, WI, USA). The microarrays were hybridized in Nimblegen hybridization buffer/hybridization component A in a hybridization chamber (Hybridization System-Nimblegen Systems, Inc., Madison, WI, USA). For array hybridization, the NimbleGen Promoter plus CpG Island array (Roche, Basel, Switzerland) was used.

The Arraystar Human T-UCR Microarray profiles the expression of 1,518 long non-coding RNAs (lncRNAs) and 2,261 mRNAs with transcription units (TU) that overlap UCRs in either the sense or antisense orientation. Sample RNA labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, Santa Clara, CA, USA), with minor modifications. The hybridized arrays were washed, fixed and scanned, using Agilent DNA Microarray Scanner (part no. G2505C). Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies).

Bioinformatics analysis

CpG methylation enrichment in the MHC segment and analysis for differential enrichment. The MHC gene capture sequencing segment was chr6:28477797-33448354. To search the enrichment location, we analyzed the CpG peaks in the MHC segment.

T-UCR expression in the MHC segment. To search for the location of transcripts, we analyzed T-UCR expression in the MHC segment.

Effect of the CpG methylation level and T-UCR expression level in immunological processes. We analyzed all methylated CpGs, T-UCR, and their corresponding genes, and then analyzed the related genes with regard to immunological processes. To further examine the functions of these genes, we used the Online Gene Ontology Tool EASE (<http://david.abcc.ncifcrf.gov/ease/ease1.htm>). The differentially expressed genes were classified with regard to biological processes. Gene Ontology (GO) and KEGG pathway mapping of the genes was performed using the web-accessible DAVID annotation system.

Correlation of MHC mutation with CpG methylation. To identify correlations, we calculated the data of differential CpG methylation and MHC mutation and analyzed the correlation coefficients.

Results

Capturing the number of genes and SNP loci in the MHC region. We obtained 150 genes and 27,066 SNPs by MHC gene capture and high-throughput sequencing in the patients with SLE compared with the normal controls (data not shown).

hMeDIP-chip. The 3,826 genes with CpG islands had significantly different methylation levels in the patients with SLE compared with the normal controls, as was also previously noted (11).

T-UCR microarray analysis. To identify potential differentially expressed T-UCRs, we performed fold change filtering of

Table I. Twelve CpG-methylated enrichment sites in the MHC segment.

CpG name (hg19)	Length (bp)	Control	SLE	Gene name	Location
chr6:30042918-30043500	582		1	<i>RNF39</i>	Promoter
chr6:31323946-31325211	1,265		1	<i>HLA-B</i>	Promoter
chr6:31695894-31698245	2,351		1	<i>DDAH2</i>	Promoter
chr6:31695894-31698245	2,351		1	<i>LY6G6C</i>	Promoter
chr6:31695894-31698245	2,351		1	<i>MSH5</i>	Promoter
chr6:32935896-32936792	896		1	<i>BRD2</i>	Promoter
chr6:29521110-29521833	723	1		<i>UBD</i>	Intergenic
chr6:30538983-30539487	504	1		<i>ABCF1</i>	Promoter
chr6:30684836-30685503	667	1		<i>MDC1</i>	Promoter
chr6:30684836-30685503	667	1		<i>TUBB</i>	Promoter
chr6:31548436-31549277	841	1		<i>LST1</i>	Promoter
chr6:32975684-32975926	242	1		<i>HLA-DPB2</i>	Intragenic

MHC, major histocompatibility complex; Control, healthy control subjects; SLE, systemic lupus erythematosus patients.

Table II. *HLA-B* promoter region and *HLA-DPB2* intragenic region in patients with SLE.

HLA-B promoter region (chr6:31323946-31325211, 1265 bp)

CGAAGTCCCAGGTCCCGGACGGGGCTCTCAGGGTCTCAGGCTCCGAGGGCCGCGTCTGCAATGGGGAGGCGCAG
CGTTGGGGATTCCCCACTCCCCTGAGTTTCACTTCTTCTCCCAACTTGTGTCTGGGTCTCTTCTCCAGGATACTCGTG
ACGCGTCCCCACTTCCCCTCCATTGGGTATTGGATATCTAGAGAAGCCAATCAGCGTCGCCGCGGTCCAGTTC
TAAAGTCCCCACGCACCCACCCGACTCAGAGTCTCCTCAGACGCCGAGATGCTGGTCATGGCGCCCCGAACCGT
CCTCCTGCTGCTCTCGGCGGCCCTGGCCCTGACCGAGACCTGGGCCGGTGAGTGCGGGTTCGGGAGGGAAATGGC
CTCTGCCGGGAGGAGCGAGGGGACCGCAGGCGGGGGCGCAGGACCTGAGGAGCCGCGCCGGGAGGAGGGTTCGG
GCGGGTCTCAGCCCCCTCCTACCCCCAGGCTCCCACTCCATGAGGTATTTCTACACCTCCGTGTCCCGGCCCGGCC
GCGGGGAGCCCCGCTTCATCTCAGTGGGCTACGTGGACGACACCCAGTTTCGTGAGGTTTCGACAGCGACGCCGCG
AGTCCGAGAGAGGAGCCGCGGGCGCCGTGGATAGAGCAGGAGGGGCCGAGTATTGGGACCGGAACACACAGA
TCTACAAGGCCAGGCACAGACTGACCGAGAGAGCCTGCGGAACCTGCGCGGCTACTACAACCAGAGCGAGGCC
GGTGAGTGACCCCGGCCCGGGGCGCAGGTACGACTCCCCATCCCCACGTACGGCCCGGGTTCGCCCCGAGTCTC
CGGGTCCGAGATCCGCTCCCTGAGGCGCGGGACCCGCCAGACCTCGACCGGCGAGAGCCCCAGGCGCGTT
TACCCGTTTTCATTTTCAGTTGAGGCCAAAATCCCCGCGGGTTGGTTCGGGGCGGGGCGGGGCTCGGGGGACTGGG
CTGACCGCGGGGCGGGGCCAGGGTCTCACACCTCCAGAGCATGTACGGCTGCGACGTGGGGCCGGACGGGCG
CCTCCTCCGCGGGCATGACCACTACGCCTACGACGGCAAGGATTACATCGCCCTGAACGAGGACCTGCGCTCCTG
GACCGCCGCGGACACGGCGGCTCAGATACCCAGCGCAAGTGGGAGGCGGCCCGTGAGGCGGAGCAGCGGAGA
GCCTACCTGGAGGGCGAGTGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAAGGACAAGCTGGAGCGCGC

HLA-DPB2 intragenic region (chr6:32975684-32975926, 242 bp)

CGAGGCCGTGTGGCGTCTGCCTGAGTTTGGTGACTTTGCCCGCTTTGACCCGCGAGGGCGGGCTGGCCGGCATCGC
CGCAATCAAAGCCCATCTGGACATCCTGGTGGAGCGCTCCAACCGCAGCAGAGCCATCAACGGTACCGGCCCTCC
CTCTGCCACCCAGTCAGGCGGGAAGGTCCAGAGAACTTCTCCAGTTCCTAGGCTCCCATCACTCTGGGGCG
CGCTCTCAGCGCCCGCGC

SLE, systemic lupus erythematosus; HLA, human leukocyte antigen.

the SLE patients compared with the normal controls. We found a signature of 8 upregulated T-UCRs and 29 downregulated T-UCRs (data not shown).

CpG peak in the MHC segment. To search for enrichment locations, we analyzed CpG peaks in the MHC segment. The results indicated the enrichment of 12 CpG-methylated

sites (Table I), with 6 in the patients with SLE and 6 in the normal controls. One CpG-methylated enrichment site was found to be located in the *HLA-B* promoter region (chr6:31323946-31325211, 1,265 bp) (Table II) in the patients with SLE; another site was located in the *HLA-DPB2* intragenic region (chr6:32975684-32975926, 242 bp) (Table II) in the control group.

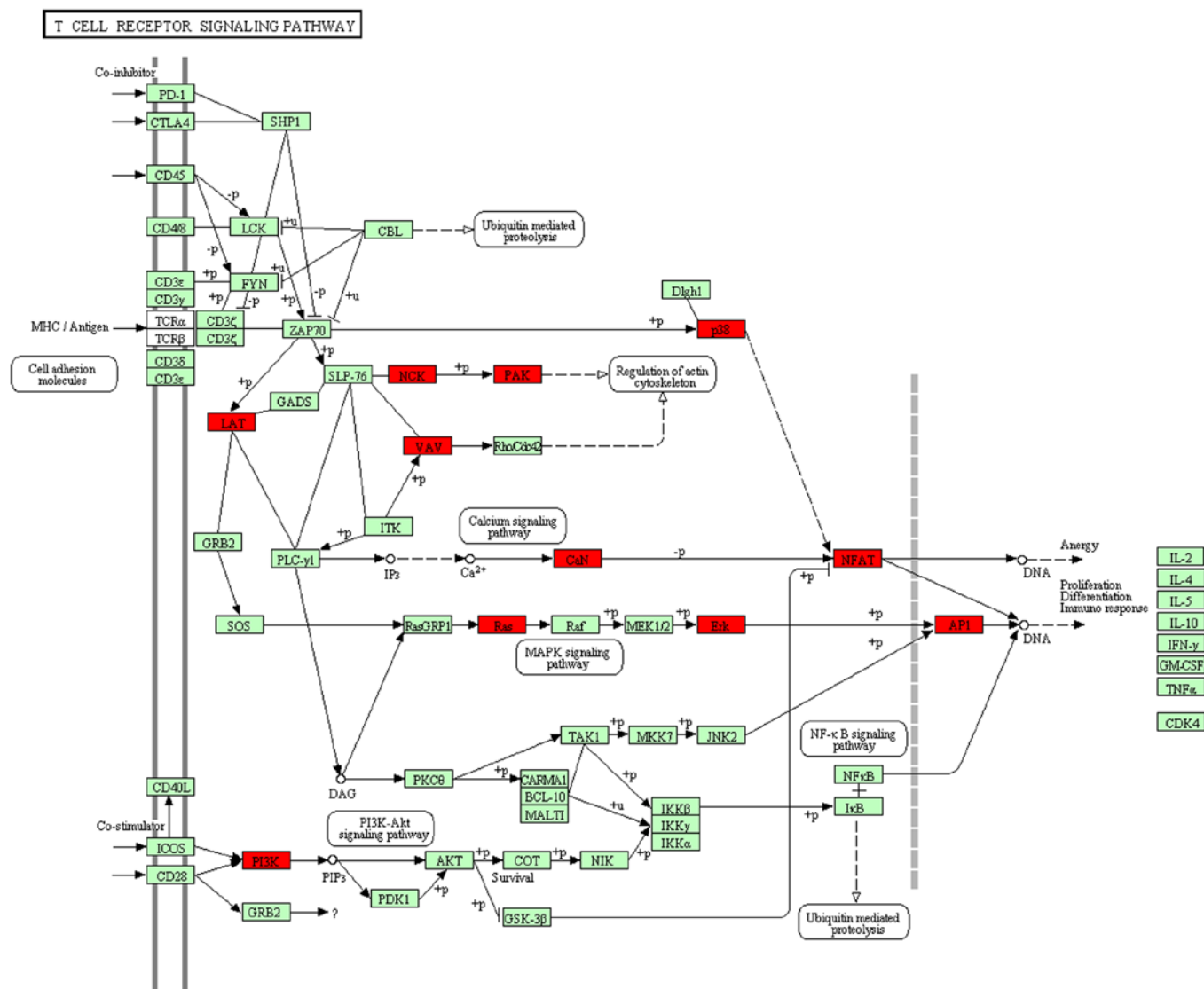


Figure 1. The immune-correlated process KEGG pathway hsa04660 T cell receptor signaling pathway.

T-UCR expression in the MHC segment. In the present study, we analyzed the expression of all T-UCRs; however, UCR-overlapping and UCR-proximal genes were not discovered in the MHC segment.

Effect of the CpG methylation level on immunological processes. We annotated corresponding CpG-methylated genes in the patients with SLE with GO schemes using the DAVID gene annotation tool. The genes produced a total of 97 GO terms in the patients with SLE (Table III). However, no significant enrichment was found for immune-correlated process GO terms, such as GO:0006955 - immune response (48 genes: *TNFAIP8 L2*, *ITGAL*, *GALNT2*, *YWHAZ*, *LST1*, *TOLLIP*, *IFITM2*, *IFITM3*, *SUSD2*, *TLR2*, *NLRX1*, *VTN*, *TLR5*, *CX3CL1*, *PYDC1*, *FTH1*, *IGF1R*, *TUBB*, *IL2RG*, *CFD*, *SPON2*, *APLN*, *SPN*, *DNAJA3*, *POLL*, *TRPM4*, *DBNL*, *IL18R1*, *SMAD6*, *EOMES*, *CNPY3*, *STXBP2*, *POLR3A*, *HLA-B*, *VAV1*, *WAS*, *CD1D*, *LAT*, *CYBA*, *PRELID1*, *GPI*, *SARM1*, *ULBP1*, *TGFB3*, *ADAM17*, *TCF12*, *ICOSLG*, *TNFAIP1*, $P=0.999999$, $FDR >0.05$).

In addition, we obtained 3 KEGG pathways for genes in patients with SLE (Table IV), although no significant enrichment was found for immune-correlated process KEGG pathways, such as hsa04660: T cell receptor signaling pathway (Fig. 1; 15 genes: *PIK3CG*, *HRAS*, *VAV1*, *LOC407835*, *MAPK1*, *LAT*, *MAPK12*, *NCK1*, *PAK4*, *JUN*, *NFAT5*, *PPP3CB*, *CHP*, *PIK3R3*, *NFATC1*, $P=0.406339$, $FDR >0.05$).

Effect of T-UCR expression levels on immunological processes. We also annotated T-UCR-corresponding genes with GO schemes using the DAVID gene annotation tool. The genes produced total 43 GO terms in patients with SLE (Table V); however, no significant enrichment was found for immune-correlated process GO terms. In addition, we did not obtain immune-correlated process KEGG pathways for the genes in patients with SLE, i.e., there was no significant enrichment.

Correlation between MHC mutation and CpG methylation. In this study, we found 4 SNPs in the CpG promoter (chr6:31323946-

Table III. GO term annotations of corresponding CpG-methylated genes in SLE patients.

GO term	Gene count	P-value	FDR
GO:0030182 - neuron differentiation	113	1.39E-13	2.58E-10
GO:0051252 - regulation of RNA metabolic process	319	2.37E-10	4.41E-07
GO:0006355 - regulation of transcription, DNA-dependent	313	2.50E-10	4.66E-07
GO:0045449 - regulation of transcription	429	8.22E-10	1.53E-06
GO:0000904 - cell morphogenesis involved in differentiation	66	3.46E-09	6.45E-06
GO:0048666 - neuron development	82	1.08E-08	2.01E-05
GO:0007409 - axonogenesis	55	1.18E-08	2.19E-05
GO:0006350 - transcription	349	2.66E-08	4.96E-05
GO:0048667 - cell morphogenesis involved in neuron differentiation	57	3.36E-08	6.26E-05
GO:0000902 - cell morphogenesis	83	5.01E-08	9.34E-05
GO:0007389 - pattern specification process	66	1.46E-07	2.73E-04
GO:0048812 - neuron projection morphogenesis	56	1.71E-07	3.18E-04
GO:0031175 - neuron projection development	63	3.43E-07	6.39E-04
GO:0048858 - cell projection morphogenesis	61	3.52E-07	6.56E-04
GO:0032989 - cellular component morphogenesis	87	4.22E-07	7.86E-04
GO:0032990 - cell part morphogenesis	62	7.71E-07	0.001437153
GO:0006357 - regulation of transcription from RNA polymerase II promoter	138	1.20E-06	0.002241345
GO:0030030 - cell projection organization	80	1.79E-06	0.003344582
GO:0003002 - regionalization	50	2.55E-06	0.004748657
GO:0021953 - central nervous system neuron differentiation	18	2.92E-06	0.005449045
GO:0030900 - forebrain development	41	4.82E-06	0.008984544
GO:0048663 - neuron fate commitment	18	6.52E-06	0.012150091
GO:0048598 - embryonic morphogenesis	67	1.19E-05	0.022252245
GO:0021954 - central nervous system neuron development	15	1.47E-05	0.02743134
GO:0045944 - positive regulation of transcription from RNA polymerase II promoter	77	1.68E-05	0.031223255
GO:0007411 - axon guidance	31	1.93E-05	0.035915973
GO:0045165 - cell fate commitment	37	2.06E-05	0.038453597
GO:0021872 - generation of neurons in the forebrain	10	2.17E-05	0.040470383
GO:0045893 - positive regulation of transcription, DNA-dependent	93	2.77E-05	0.051580919
GO:0051254 - positive regulation of RNA metabolic process	93	3.85E-05	0.071644433
GO:0006928 - cell motion	92	3.98E-05	0.074087062
GO:0007423 - sensory organ development	52	4.33E-05	0.08062919
GO:0007169 - transmembrane receptor protein tyrosine kinase signaling pathway	51	4.81E-05	0.089665676
GO:0021879 - forebrain neuron differentiation	9	4.99E-05	0.092920613
GO:0045935 - positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	114	6.21E-05	0.115713935
GO:0051173 - positive regulation of nitrogen compound metabolic process	117	6.28E-05	0.116921469
GO:0060284 - regulation of cell development	47	8.31E-05	0.154746845
GO:0045941 - positive regulation of transcription	104	9.33E-05	0.173822702
GO:0050767 - regulation of neurogenesis	40	1.00E-04	0.186897834
GO:0045664 - regulation of neuron differentiation	34	1.11E-04	0.206978981
GO:0010628 - positive regulation of gene expression	106	1.20E-04	0.223869384
GO:0010557 - positive regulation of macromolecule biosynthetic process	117	1.23E-04	0.228227922
GO:0016192 - vesicle-mediated transport	105	1.36E-04	0.253359896
GO:0051960 - regulation of nervous system development	44	1.46E-04	0.271915907
GO:0031328 - positive regulation of cellular biosynthetic process	121	1.62E-04	0.302155781
GO:0009891 - positive regulation of biosynthetic process	122	1.98E-04	0.368213146
GO:0007167 - enzyme linked receptor protein signaling pathway	68	2.08E-04	0.386237807
GO:0045892 - negative regulation of transcription, DNA-dependent	70	2.33E-04	0.432663991
GO:0030817 - regulation of cAMP biosynthetic process	27	3.08E-04	0.572491129
GO:0002009 - morphogenesis of an epithelium	27	3.08E-04	0.572491129

Table III. Continued.

GO term	Gene count	P-value	FDR
GO:0030902 - hindbrain development	19	3.48E-04	0.647196595
GO:0021537 - telencephalon development	20	3.49E-04	0.648170377
GO:0021761 - limbic system development	13	3.51E-04	0.6529104
GO:0051253 - negative regulation of RNA metabolic process	70	3.83E-04	0.7106764
GO:0045934 - negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	93	3.88E-04	0.7213528
GO:0010604 - positive regulation of macromolecule metabolic process	144	3.97E-04	0.737772867
GO:0030814 - regulation of cAMP metabolic process	27	4.29E-04	0.795879925
GO:0009792 - embryonic development ending in birth or egg hatching	65	5.31E-04	0.984899092
GO:0008285 - negative regulation of cell proliferation	69	5.90E-04	1.094286156
GO:0051172 - negative regulation of nitrogen compound metabolic process	93	6.13E-04	1.136642775
GO:0009890 - negative regulation of biosynthetic process	101	6.30E-04	1.168027793
GO:0010558 - negative regulation of macromolecule biosynthetic process	97	6.65E-04	1.231652838
GO:0009952 - anterior/posterior pattern formation	33	6.76E-04	1.252734624
GO:0031327 - negative regulation of cellular biosynthetic process	99	6.87E-04	1.273119736
GO:0043583 - ear development	25	6.92E-04	1.282237143
GO:0051339 - regulation of lyase activity	26	7.49E-04	1.385942497
GO:0048732 - gland development	32	7.50E-04	1.387778635
GO:0045761 - regulation of adenylate cyclase activity	25	8.13E-04	1.504562886
GO:0022037 - metencephalon development	13	9.00E-04	1.663443567
GO:0048568 - embryonic organ development	38	9.18E-04	1.697487359
GO:0021766 - hippocampus development	10	9.31E-04	1.721218707
GO:0016481 - negative regulation of transcription	83	9.44E-04	1.744715102
GO:0030808 - regulation of nucleotide biosynthetic process	27	0.001237	2.280679952
GO:0030802 - regulation of cyclic nucleotide biosynthetic process	27	0.001237	2.280679952
GO:0010629 - negative regulation of gene expression	89	0.001288	2.373367738
GO:0031279 - regulation of cyclase activity	25	0.001292	2.380392647
GO:0035107 - appendage morphogenesis	25	0.001292	2.380392647
GO:0035108 - limb morphogenesis	25	0.001292	2.380392647
GO:0021543 - pallium development	15	0.00132	2.432197518
GO:0014031 - mesenchymal cell development	16	0.001327	2.444625723
GO:0048762 - mesenchymal cell differentiation	16	0.001327	2.444625723
GO:0060562 - epithelial tube morphogenesis	19	0.001468	2.701431115
GO:0010941 - regulation of cell death	134	0.001518	2.791424456
GO:0035295 - tube development	45	0.001562	2.870764354
GO:0048705 - skeletal system morphogenesis	27	0.001632	2.999117627
GO:0060485 - mesenchyme development	16	0.001648	3.027543666
GO:0030799 - regulation of cyclic nucleotide metabolic process	27	0.001868	3.424511611
GO:0001709 - cell fate determination	12	0.001898	3.478414435
GO:0043009 - chordate embryonic development	62	0.001904	3.49042025
GO:0016331 - morphogenesis of embryonic epithelium	17	0.001959	3.588514741
GO:0042127 - regulation of cell proliferation	129	0.002101	3.843962583
GO:0048736 - appendage development	25	0.002293	4.187971146
GO:0060173 - limb development	25	0.002293	4.187971146
GO:0051349 - positive regulation of lyase activity	17	0.002374	4.334185806
GO:0035239 - tube morphogenesis	29	0.002504	4.564895517
GO:0043067 - regulation of programmed cell death	132	0.002508	4.573381853
GO:0017145 - stem cell division	6	0.002742	4.98879198

A P-value <0.005 was considered to indicate a statistically significant difference. The false discovery rate (FDR) of a set of predictions is the expected percentage of false predictions in the set of predictions; an FDR <5% may be quite meaningful. SLE, systemic lupus erythematosus.

Table IV. KEGG pathway annotation of corresponding CpG-methylated genes in patients with SLE.

Pathways	Gene count	P-value	FDR
hsa05200: pathways in cancer	60	5.25E-04	0.6471034
hsa04916: melanogenesis	23	0.002387	2.9086362
hsa05217: basal cell carcinoma	15	0.003991	4.8195711

A P-value <0.005 was considered to indicate a statistically significant difference. The false discovery rate (FDR) of a set of predictions is the expected percentage of false predictions in the set of predictions; an FDR <5% may be quite meaningful. SLE, systemic lupus erythematosus.

31325211) of *HLA-B* and 2 SNPs in chr6:29521110-29521833 in the control patients (Table VI).

Discussion

The first genetic factors to be identified as important in the pathogenesis of SLE were those of the MHC on chromosome 6. It is now widely accepted that MHC genes constitute a part of the genetic susceptibility to SLE (12). However, previous studies on SLE have lacked statistical power and the genetic resolution to fully define the influences of the MHC (13,14). In this study, we attempted to identify MHC, CpG methylation and T-UCR to reveal the potential mechanisms responsible for the development of SLE using a novel and combinatorial approach involving MHC gene capture technology, hMedIP-chip, T-UCR microarray and bioinformatics analysis. A total of 27,066 SNPs were detected and thus these may be involved in SLE. Moreover, we integrated the datasets and identified 6 of the most important SNPs in SLE. Our next step is to perform research on the function of these SNPs.

HLA antigens and genes have long been reported to be associated with SLE susceptibility in a number of populations (15). With advances in technologies, such as genome-wide association studies, a number of newly discovered SLE-associated SNPs have been reported in recent years. These include *HLA-DRB1/HLA-DQA1* rs9271366 and *HLA-DQB1/HLA-DQA2* rs9275328 (15). Previously, a meta-analysis of the MHC region in patients with SLE was performed to determine associations with both SNPs and classical HLA alleles. The results of a conditional analysis and model choice with the use of the Bayesian information criterion indicated that the best model for SLE association includes both classical loci (*HLA-DRB1*03:01*, *HLA-DRB1*08:01* and *HLA-DQA1*01:02*) and 2 SNPs, rs8192591 (in class III and upstream of NOTCH4) and rs2246618 (MICB in class I) (16). Single-marker analyses have revealed strong signals for SNPs within several MHC regions, as well as for *HLA-DRB1*. The most strongly associated DRB1 alleles are *0301, *1401 and *1501, and the MHC region SNP demonstrating the strongest evidence of an association with SLE is rs3117103 (3). These results delineate with high resolution several MHC regions which contribute independently to the risk of developing SLE. In the present study, we integrated the MHC and CpG-methylated datasets, and the results indicated CpG methylation enrichment at 6 sites: *RNF39*, *HLA-B*, *DDAH2*, *LY6G6C*, *MSH5* and *BRD2* in the MHC regions of SLE. These genes play important

roles in various immune diseases, including SLE. SNPs in the region of the *RNF39* gene have been found to be associated with the disease course of HIV-1 (17,18). Behcet's disease is a chronic inflammatory autoimmune disease that is strongly associated with *HLA-B*51* and *-A26*. It has previously been suggested that *RNF39* is involved in the etiology of Behcet's disease (19). The MHC region is suspected to host susceptibility loci for HIV-related Kaposi's sarcoma, involving the rs1065356 (*LY6G6C*) and rs3749953 (*MSH5-SAPCD1*) (20). *MSH5* has been found to be mutated in patients with common variable immunodeficiency (21). In a previous study, a significant increase in the frequency of *HLA-A*01*, *A*03*, *A*11*, *A*23*, *A*26*, *A*69*, *HLA-B*27*, *B*40*, *B*49*, *B*51*, *B*52*, *B*53*, *B*54*, *B*95*, *HLA-DRB1*01*, *DRB1*03*, *DRB1*11* and *DRB1*14* was observed in SLE patients, indicating a positive association of these alleles with SLE. By contrast, *HLA-A*24*, *A*29*, *A*31*, *A*34*, *A*68*, *A*92*, *HLA-B*18* and *HLA-DRB1*12* were found to be decreased in the patient group compared to the controls, indicating a negative association of these alleles with SLE. Thus, it was concluded that SLE is associated with certain MHC alleles, such as *HLA-B*, in the Pakistani population (12). As the results of the present study indicated the enrichment of one CpG methylation site located in the *HLA-B* promoter region, *HLA-B* may indeed play an important role in the pathogenesis of SLE. Moreover, we found 4 SNPs (rs1050683, rs12697943, rs17881210 and rs1065378) in the CpG region of the *HLA-B* promoter and 2 SNPs (rs17184255 and rs16895070) in MHC regions. These SNPs were significantly associated with an increased risk of developing SLE.

SLE is an autoimmune disease with known genetic, epigenetic, and environmental risk factors. Epigenetic events play a central role in the priming, differentiation and subset determination of T lymphocytes. CpG-DNA methylation and post-translational modifications to histone tails are the two most well-accepted epigenetic mechanisms. Furthermore, the involvement of epigenetic mechanisms in the pathogenesis of SLE has been suggested by the development of lupus-like symptoms in individuals who are treated with procainamide or hydralazine, resulting in a reduction in CpG-DNA methylation (22). In SLE, global CpG-DNA hypomethylation correlates with disease activity. A number of cytokine genes are overexpressed in CD4⁺ T lymphocytes from patients with SLE in a chromatin-dependent manner, including IL-6 (23). Region-specific histone acetylation in certain tissues is associated with increased disease activity, whereas histone acetylation in other regions has protective effects. In SLE, acetylation of the TNF

Table V. GO term annotation of T-UCR corresponding genes in SLE patients.

GO term	Gene count	P-value	FDR
GO:0008380 - RNA splicing	35	1.24E-11	2.15E-08
GO:0006397 - mRNA processing	37	2.04E-11	3.55E-08
GO:0016071 - mRNA metabolic process	38	3.00E-10	5.22E-07
GO:0006396 - RNA processing	47	6.45E-10	1.12E-06
GO:0006357 - regulation of transcription from RNA polymerase II promoter	55	1.75E-09	3.04E-06
GO:0045449 - regulation of transcription	129	1.15E-08	1.99E-05
GO:0051252 - regulation of RNA metabolic process	97	5.09E-08	8.85E-05
GO:0045935 - positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	44	7.25E-07	0.001261
GO:0051254 - positive regulation of RNA metabolic process	37	9.25E-07	0.00161
GO:0006355 - regulation of transcription, DNA-dependent	91	1.01E-06	0.00175
GO:0010558 - negative regulation of macromolecule biosynthetic process	40	1.07E-06	0.001865
GO:0000398 - nuclear mRNA splicing, via spliceosome	19	1.20E-06	0.002082
GO:0000377 - RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	19	1.20E-06	0.002082
GO:0000375 - RNA splicing, via transesterification reactions	19	1.20E-06	0.002082
GO:0045944 - positive regulation of transcription from RNA polymerase II promoter	31	1.60E-06	0.002782
GO:0051173 - positive regulation of nitrogen compound metabolic process	44	1.68E-06	0.002914
GO:0031327 - negative regulation of cellular biosynthetic process	40	1.97E-06	0.003434
GO:0045893 - positive regulation of transcription, DNA-dependent	36	2.06E-06	0.003575
GO:0045941 - positive regulation of transcription	40	2.26E-06	0.00393
GO:0009890 - negative regulation of biosynthetic process	40	3.34E-06	0.005812
GO:0048598 - embryonic morphogenesis	27	3.46E-06	0.006015
GO:0045934 - negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	37	3.86E-06	0.00672
GO:0006350 - transcription	101	4.00E-06	0.006958
GO:0010628 - positive regulation of gene expression	40	4.62E-06	0.008041
GO:0051172 - negative regulation of nitrogen compound metabolic process	37	5.24E-06	0.009119
GO:0031328 - positive regulation of cellular biosynthetic process	44	8.06E-06	0.014026
GO:0051253 - negative regulation of RNA metabolic process	29	8.35E-06	0.014517
GO:0010605 - negative regulation of macromolecule metabolic process	46	8.87E-06	0.015419
GO:0010604 - positive regulation of macromolecule metabolic process	51	1.09E-05	0.018888
GO:0009891 - positive regulation of biosynthetic process	44	1.15E-05	0.020058
GO:0016481 - negative regulation of transcription	33	1.61E-05	0.027966
GO:0045892 - negative regulation of transcription, DNA-dependent	28	1.72E-05	0.029862
GO:0010557 - positive regulation of macromolecule biosynthetic process	41	3.08E-05	0.053493
GO:0010629 - negative regulation of gene expression	34	4.16E-05	0.072327
GO:0000122 - negative regulation of transcription from RNA polymerase II promoter	21	2.37E-04	0.412261
GO:0048568 - embryonic organ development	16	2.97E-04	0.515968
GO:0016055 - Wnt receptor signaling pathway	13	8.69E-04	1.501407
GO:0030900 - forebrain development	14	8.89E-04	1.535269
GO:0043009 - chordate embryonic development	22	0.001544	2.651627
GO:0009792 - embryonic development ending in birth or egg hatching	22	0.001718	2.946609
GO:0046907 - intracellular transport	35	0.002355	4.018647
GO:0015931 - nucleobase, nucleoside, nucleotide and nucleic acid transport	11	0.002648	4.507588
GO:0048562 - embryonic organ morphogenesis	12	0.002821	4.795051

A P-value <0.005 was considered significant. The false discovery rate (FDR) of a set of predictions is the expected percent of false predictions in the set of predictions; an FDR <5% may be quite meaningful. SLE, systemic lupus erythematosus; T-UCR, transcribed ultra-conserved regions.

Table VI. Six CpG-methylated SNPs of the MHC segment in patients with SLE.

Chromosome segment	SNP	Gene	Location
chr6:31324019	rs1050683	HLA-B	Exonic
chr6:31324057	rs12697943	HLA-B	Exonic
chr6:31324448	rs17881210	HLA-B	Intronic
chr6:31324633	rs1065378	HLA-B	Exonic
chr6:29521289	rs17184255		Intergenic
chr6:29521557	rs16895070		Intergenic

MHC, major histocompatibility complex; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism.

promoter in monocytes is associated with increased monocyte maturation and cytokine expression (24). Thus, a better understanding of the molecular events that contribute to epigenetic alterations and subsequent immune imbalance is essential for the establishment of disease biomarkers and the identification of potential therapeutic targets (22). To assess the role of DNA methylation in SLE, researchers collected CD4⁺ T-cells, CD19⁺ B-cells, and CD14⁺ monocytes, and performed a genome-wide DNA methylation analysis with the use of IlluminaMethylation 450 microarrays. Interferon hypersensitivity was apparent in memory, naïve and regulatory T-cells, suggesting that this epigenetic state in lupus patients is established in progenitor cell populations. These cell type-specific effects are consistent with the disease-specific changes in the composition of the CD4⁺ population and suggest that shifts in the proportion of CD4⁺ subtypes can be monitored at CpGs with subtype-specific DNA methylation patterns (25). In the present study, we annotated the corresponding CpG-methylated genes using the DAVID gene annotation tool. However, immune-correlated process GO terms, such as GO:0006955-immune response, and KEGG pathways, such as hsa04660-T-cell receptor (TCR) signaling pathway exhibited no significant enrichment. The GO analysis did reveal that the 'theme' immune response (GO:0006955), which is known to be affected by anti-TNF treatment in the inflammatory tissue of rheumatoid arthritis patients, was significantly over-represented (26). Regardless, it is relevant to note in our context that our GO analysis identified immune functions as potentially relevant mechanisms. The activation of T lymphocytes is a key event for an efficient response of the immune system (hsa04660-TCR signaling pathway) and requires the involvement of the TCR as well as costimulatory molecules, such as CD28. The engagement of these receptors through interaction with a foreign antigen is associated with MHC molecules (27), and our findings may thus facilitate the selection of better target molecules for further studies. The findings of the present study may also aid future research by providing details of new pathways to be studied using a more focused approach, confirmation at the protein level and emphasis of the clinical significance.

lncRNAs are transcripts longer than ~200 nucleotides with little or no protein-coding capacity (28). Research has

shown that lncRNAs play important roles in disease development and are associated with a number of human diseases, such as cancer, Alzheimer's disease and heart disease (29). T-UCR transcripts are a novel class of lncRNAs transcribed from UCRs, a class of 481 non-coding sequences located in both intra- and inter-genic regions of the genome. UCRs are absolutely conserved (100%) between the orthologous regions of the human, rat, and mouse genomes and are actively transcribed (30,31). It has recently been proven in cancer systems that differentially expressed T-UCRs alter the functional characteristics of malignant cells. Indeed, recent data suggest that T-UCRs are altered at the transcriptional level in human tumorigenesis and that the aberrant T-UCR expression profiles can be used to differentiate human cancer types (31,32). Researchers observed that DNA hypomethylation induces T-UCR silencing in cancer cells, and the analysis of a large set of primary human tumors demonstrated that the hypermethylation of the described T-UCR CpG islands is a common event in the various tumor types (33). In the present study, we integrated the MHC and T-UCR datasets and examined the expression levels of T-UCR in the MHC segment by T-UCR microarray. We annotated the T-UCR corresponding genes using the DAVID gene annotation tool. However, no significant enrichment was found for immune-correlated process GO terms and KEGG pathways. Thus, T-UCR expression levels did not correlate with the commonly used clinicopathological features of the patients with SLE.

Taken together, in the present study, we identified 6 of the most important SNPs (rs1050683, rs12697943, rs17881210, rs1065378, rs17184255 and rs16895070) in patients with SLE. The present study indicates that SNPs in the MHC segment are potential biomarkers and are likely factors which are involved in the pathogenesis of SLE. However, further studies are required to investigate the mechanisms through which polymorphisms in this region lead to the development of SLE. A major advantage of combining multiple levels of measurement is the ability to dissect mechanisms not apparent in a single dimension. The integration of MHC, CpG methylation, and T-UCR datasets is a powerful strategy for understanding SLE biology. Our findings provide insight into the potential contribution of anomalously regulated SNPs to the abnormalities in SLE and may aid in the structuring of antenatal diagnostic biomarkers of SLE, as well as in obtaining novel therapeutic targets which can be used in the treatment of patients with SLE. Moreover, our study of SNPs may aid in the development of novel methods which may prove to be useful for treating and preventing other diseases.

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