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Epigenome-wide association study of peripheral blood mononuclear cells in systemic lupus erythematosus: Identifying DNA methylation signatures associated with interferon-related genes based on ethnicity and SLEDAI

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ABSTRACT

Systemic lupus erythematosus (SLE or lupus) is a heterogeneous autoimmune disease characterized by the involvement of multiple organs and the production of antinuclear antibodies. DNA methylation plays an important role in the pathogenesis of lupus. We have performed an epigenome-wide DNA methylation study in lupus and healthy control (non-lupus) subjects to identify epigenetic patterns in lupus characterized ethnicity and SLE disease activity index (SLEDAI). A total of fifty-seven lupus patients (39 African American (AA) and 18 European American (EA)) and 33 healthy controls (17 AA and 16 EA) were studied. Differential DNA methylation between lupus patients and controls was assessed for approximately 485,000 CpG sites across the genome. We identified 41 differentially methylated sites (associated with 30 genes) between lupus and control s subjects, 85% of which were hypomethylated. Significant hypomethylation of differentially methylated sites was associated with several interferon-related genes, including *MX1*, *IFI44L*, *PARP9*, *DT3XL*, *IFIT1*, *IFI44*, *RSAD2*, *PLSCR1*, and *IRF7*. Several of these associated genes were also hypomethylated in comparisons between AA lupus and AA non-lupus subjects and between lupus patients with SLEDAI > 6 and non-lupus subjects. Our analysis of gene expression data through RT-PCR confirmed these findings. Thus, the results indicate epigenetics susceptibility in lupus, which may be associated with SLEDAI score and ethnicity. In addition, our findings support the importance of the Type 1 interferon pathway in lupus pathogenesis.

1. Introduction

Systemic lupus erythematosus (SLE or lupus) is a heterogeneous and chronic relapsing autoimmune disease characterized by the involvement of multiple organs and the production of antinuclear antibodies [1,2]. Although the etiology of the disease is not completely understood, several environmental triggers, along with genetic factors, are thought to contribute to the pathogenesis of the disease [1–3]. Environmental factors, such as viral infection or drugs, can lead to lupus through DNA methylation alterations [3]. DNA methylation modifications can act as transcriptional regulators depending on where they occur in the genome. Studies have shown that DNA methylation in CpGs in promoter regions is associated with gene silencing [4], while DNA methylation in CpG shores and shelves is associated with tissue-specific DNA methylation [5,6].

Several targeted and global lupus DNA methylation studies have been performed. Targeted studies of specific leucocytes and T-cells subsets from patients with lupus demonstrated hypomethylation and overexpression of specific genes related to autoimmune disease [7–9]. This trend was also observed in global DNA methylation studies utilizing unfractionated blood [10,11]. Interferon signaling has been one of the most commonly identified pathways regardless of type of study, and several interferon related genes have been identified as key biomarkers of lupus [7–11].

Despite many advances in lupus research, health disparities continue to exist. The occurrence of lupus in women of color, (e.g., African Americans (AA)s, Hispanics, Asians, Alaska Natives, Native Americans, and Native Hawaiians or other Pacific Islanders), is three to four times

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higher than European Americans (EA) [12–14]. Studies have demonstrated that African Americans experience younger age of disease onset and higher disease severity and outcome when compared to European Americans. At the time of lupus diagnosis, AA lupus patients are more likely to have multi-organ system involvement, more active disease, and higher frequencies of auto-antibodies compared to EA lupus patients [13,15,16]. DNA methylation has been shown to contribute to some of the phenotypic differences between individuals and ethnicities [12]. Thus, studying epigenetic changes is integral when investigating autoimmune diseases, such as lupus.

In this study we utilized peripheral blood mononuclear cells (PBMC) to identify epigenetic changes associated with the inflammatory environment in lupus based on ethnicity and SLEDAI score. We performed a genome-wide DNA methylation study in lupus patients of AA and EA descent (with varying SLEDAI scores), and compared their methylation pattern with normal healthy controls of AA and EA descent. We also performed ancillary analysis to assess the correlation between DNA methylation and SLEDAI score for important subgroups of patients.

2. Methods and materials

2.1. Lupus and non-lupus (NL) patients

The subjects in this study were women who were enrolled in an approved study at the Brody School of Medicine at East Carolina University [17]. Therefore, existing diagnostic specimens were used in this study. Because of the use of existing diagnostic specimens from a previous approved study this project was granted an exemption from IRB (15-032T) from both the Department of Health and Human Services and the Food and Drug Administration (DHHS FDA). A total of 163 women (83 lupus and 80 non-lupus (NL) subjects) participated in the original study. Lupus diagnosis was based on SLE disease activity index (SLEDAI) score and anti-dsDNA antibody analysis. Characteristics of the cohort (age, ethnicity and SLEDAI score) are shown in Table 1.

2.2. RNA and DNA isolation

Existing DNA and RNA samples were used in this study from previous diagnostic specimens. Total RNA was extracted from peripheral blood mononuclear cells (PBMC) as previously described using PAXgene RNA kits (Qiagen, Valencia, CA) [17]. The concentration and purity of RNA samples were determined using a NanoDrop Spectrophotometer ND1000 (Thermofisher, Grand Island, NY). RNA integrity was measured using a Bio-Rad Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA); samples with an RNA integrity number (RIN) $>$ 8 were used for qRT-PCR validation. Genomic DNA was isolated from PBMC using PAXgene DNA kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. DNA concentration and integrity were determined using a NanoDrop Spectrophotometer ND1000 (Thermofisher). For DNA methylation studies, sodium bisulfite treatment was performed using EZ DNA Methylation kits (Zymo Research, Irvine, CA).

2.3. Epigenome-wide DNA methylation profiling

Epigenome-wide DNA methylation analysis was performed on 57 lupus and 33 NL subjects (55% of the cohort) using an Illumina Infinium Human Methylation450 BeadChip (450 K) array. The array targets over 485,000 CpG sites covering 99% RefSeq of genes, and provides coverage throughout the genes promoter region, 5′UTR, first exon, gene body, and 3′UTR, as well as CpG islands and their surrounding shores and shelves. Overall, the array covers 96% of CpG islands. Beta values, given by $\beta = M/(U + M + 100)$ or the ratio of methylated (M) to unmethylated (U) probes, were generated by Genome Studio software.

2.4. Differential DNA methylation analysis

Quality control was assessed using the *wateRmelon* R package. The filtering process removed 1 sample with a detection p-value $>$ 0.05 for 1% of sites, 533 sites with a bead count $<$ 3 in 5% of samples, and 2218 sites with a detection p-value $>$ 0.05 in 1% of samples. In addition, sites on the Y chromosome were removed and the rmSNP and CH function in the *DMRcate* R package [18] was used to remove known cross-reactive CpG probes [19] and probes closer than 2 nucleotides of a SNP with minor allele frequency $>$ 0.1. The preprocessed dataset consisted of 438,799 CpG sites. Following the recommendation of Marabita et al., methylation data were normalized with the QN.BMIQ approach [20]. First, between-array normalization was performed via quantile normalization. Then, beta mixture quantile normalization was implemented to correct for probe type bias (Infinium I and II) within arrays [21]. Principal component analysis (PCA) on the adjusted M-values, where $M = \log_2(\beta/(1-\beta))$, indicated that the first principal component, which accounted for 97% of the total variance, was

Table 1
Summary Characteristics of lupus and non-lupus(control) subjects.

Study		SLE patients	Non-SLE patients	*p*-value
DNA methylation Array	n	57	33	
	Age (years)	49.4 ± 2.0	55.6 ± 2.1	0.0376
	Ethnicity (n)			0.1711
	African American	39	17	
	European American	18	16	
	SLEDAI score			
	Score ≤ 6 (n = 26)	4.0 ± 0.3 (2–6)	–	
	African American	4.5 ± 0.43 (2–6)		
	European American	2.5 ± .66 (2–6)		
	Score > 6 (n = 31)	14.4 ± 1.1 (8–30)		
	African American	14.4 ± 1.1 (8–30)		
	European American	13.7 ± 1.8 (8–30)		
qRT-PCR validation	n	80	83	
	Age (years)	51.9 ± 1.6	58.4 ± 1.3	0.0016
	Ethnicity (n)			0.2674
	African American	63	58	
	European American	17	25	
	SLEDAI score			
	Score ≤ 6 (n = 52)	3.8 ± 0.2 (1–6)	–	
	Score > 6 (n = 28)	13.9 ± 1.2 (7–30)	–	

Mean ± SEM.

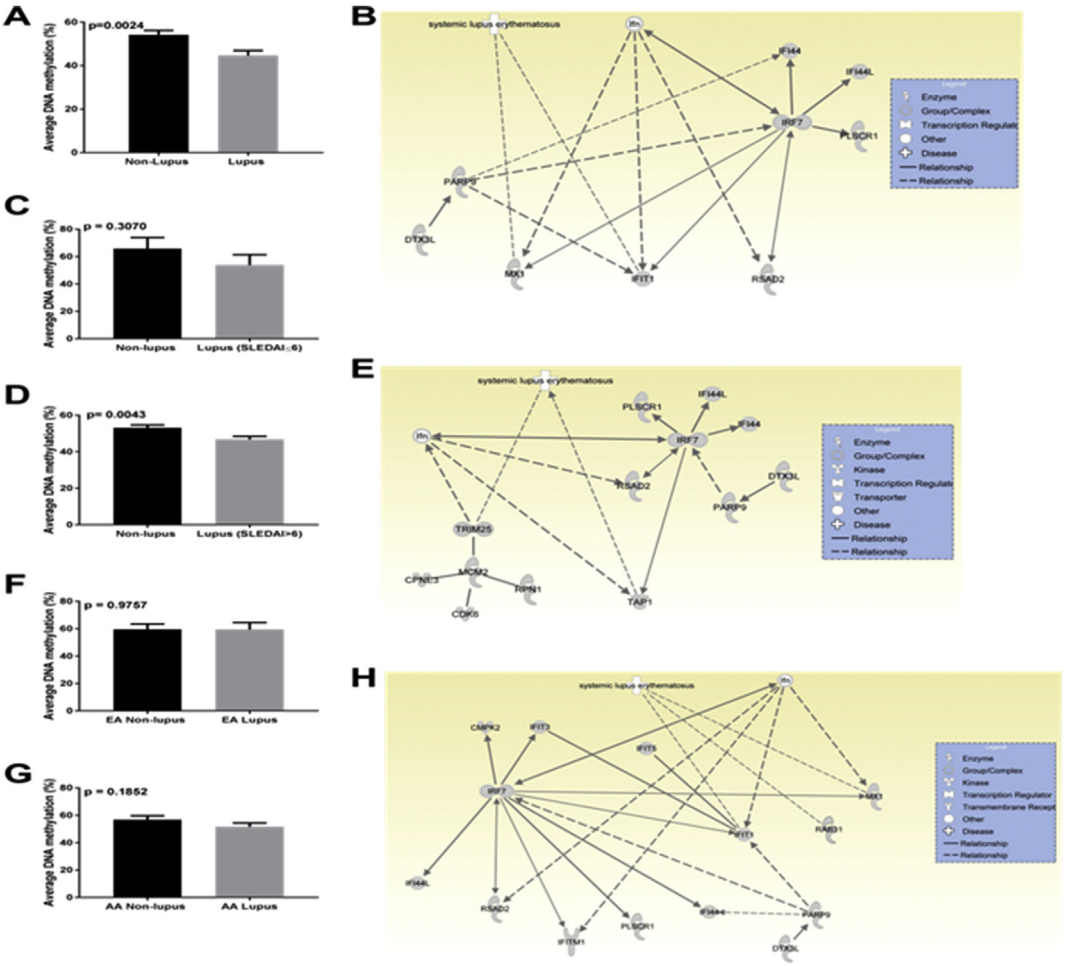

Fig. 1. DNA methylation profile of NL and lupus subjects. (A) Average DNA methylation (%) for the 41 significant CpG sites that were differentially methylated between NL and lupus subjects. Data presented as mean ± SEM. (B) Schematic illustrating the relationship between the genes associated with the differentially methylated sites (NL vs Lupus), lupus, and interferon activity. Average DNA methylation (%) for the (C) 6 significant CpG sites for the L and lupus-SLEDAI ≤ 6 subjects comparison and (D) 79 significant CpG sites for the NL and lupus-SLEDAI > 6 comparsion. Data presented as mean ± SEM. (E) Schematic illustrating the relationship between the genes associated with the differentially methylated sites (NL and lupus-SLEDAI > 6), lupus, and interferon activity. Average DNA methylation (%) for the (F) 23 significant CpG sites for the EA NL and EA lupus comparison and (G) 58 significant CpG sites for the AA NL and AA lupus comparsion. Data presented as mean ± SEM. (H) Schematic illustrating the relationship between the genes associated with the differentially methylated sites (AA NL and AA lupus), lupus, and interferon activity.

significantly associated with chip and several of the outcome variables of interest (e.g. NL vs lupus, NL vs SLEDAI > 6, etc.). Similar to findings in Buhule et al. [22], we were unable to adjust for the chip effect using Combat. After filtering a single technical replicate for two different subjects, the remaining replicates clustered together in hierarchical clustering. Mean Pearson correlation across technical replicates was 0.99 for all subjects. Hence, methylation data was averaged across replicates for each CpG site.

Differential methylation was assessed using *limma's* Bayesian framework for linear regression models [41]. The variables, *Age, Ethnicity* (AA and EA), *Score Category* (NL: SLEDAI = 0, lupus-SLEDAI ≤ 6, lupus-SLEDAI > 6), and the interaction term *Ethnicity*Score Category* were included as factors in the model. For each CpG site, we tested significance of five pairwise comparisons: 1) NL vs Lupus, 2) NL vs Lupus-SLEDAI ≤ 6, 3) NL vs Lupus-SLEDAI > 6, 4) AA-NL vs AA-Lupus, and 5) EA-NL vs EA-Lupus. An adjustment for multiple group comparisons was performed using the "global" method for the decide Tests function in *limma*. This approach adjusts for all CpG sites and multiple comparisons simultaneously using a Benjamini-Hochberg false discovery rate (FDR) [23]. CpGs with an FDR-adjusted p-values < 0.05 and a |beta difference| ≥ 0.1 were deemed significant. Prior to analysis, we performed PCA to determine whether the top principal components were associated with covariates or comparisons of interest. PC1, which accounted for 99% of the total variance, was significantly associated with *Score Category* and several of the factors formed by the pairwise comparisons of interest. Therefore, PC1 was not included in the statistical model for differential testing.

M-values were used for statistical testing and beta values were used to report/present the results. All analyses were performed in R, version 3.3.1 (http://www.r-project.com).

2.5. Pathway analysis

The *missMethyl* R package [42] was used to test for enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, in lists of significant CpG sites (global FDR < 0.05 and |beta difference| ≥ 0.1). *missMethyl* was developed specifically for methylation data to account for the different number of CpG sites per gene. Pathways with an FDR-adjusted p-value < 0.05 were considered statistically significant. In addition, Ingenuity Pathway Analysis (IPA) was performed to determine enriched canonical pathways and genes associated with lupus and interferon activity for the significant CpG sites.

Table 2
Significant hypomethylated and hypermethylated (FDR-adjusted p-values < 0.05 and |mean methylation β change| ≥ 0.1) CpG sites in lupus and non-lupus patients. Sites associated with interferon-related genes are highlighted.

	CpG Site	Chr	Gene	Methylation β value		Δβ
				Non-Lupus	Lupus	
Hypomethylated	***cg21549285***	***21***	***MX1***	***0.771***	***0.485***	***0.286***
	cg05696877	***1***	***IFI44L***	***0.620***	***0.385***	***0.235***
	cg22930808	***3***	***PARP9;DTX3L***	***0.691***	***0.462***	***0.229***
	cg00959259	***3***	***PARP9;DTX3L***	***0.564***	***0.349***	***0.215***
	cg05552874	***10***	***IFIT1***	***0.649***	***0.462***	***0.187***
	cg22862003	***21***	***MX1***	***0.650***	***0.463***	***0.186***
	cg01079652	***1***	***IFI44***	***0.953***	***0.790***	***0.164***
	cg06981309	***3***	***PLSCR1***	***0.495***	***0.333***	***0.162***
	cg06872964	***1***	***IFI44L***	***0.476***	***0.330***	***0.146***
	cg12162100	8	CPNE3	0.543	0.418	0.125
	cg10028625	3	ECT2	0.557	0.433	0.124
	cg10827754	2	–	0.531	0.408	0.124
	cg01521397	20	TAF4	0.498	0.375	0.123
	cg11639615	3	–	0.553	0.433	0.119
	cg02353916	4	LOC285550	0.511	0.392	0.119
	cg25199552	1	KDM1A	0.547	0.431	0.116
	cg06719445	14	FOXN3	0.415	0.300	0.115
	cg16788865	12	–	0.436	0.323	0.114
	cg08926253	***11***	***IRF7***	***0.613***	***0.502***	***0.112***
	cg15065340	3	TNK2	0.659	0.549	0.110
	cg23213327	***2***	***RSAD2***	***0.525***	***0.415***	***0.110***
	cg20107506	4	–	0.513	0.404	0.110
	cg17301248	12	NAA25	0.441	0.331	0.110
	cg00682263	15	MEGF11	0.437	0.329	0.108
	cg04739200	6	MYB	0.512	0.406	0.106
	cg01054652	11	–	0.585	0.479	0.106
	cg12213414	2	LYG2	0.621	0.517	0.104
	cg12401842	12	CLEC4A	0.484	0.380	0.104
	cg09697978	2	PRKD3	0.466	0.363	0.103
	cg26928972	3	CSTA	0.473	0.371	0.102
	cg04196862	17	HOXB5;LOC404266	0.494	0.393	0.101
	cg21648069	12	ERC1	0.433	0.332	0.101
	cg23599224	4	–	0.548	0.446	0.101
	cg17069914	3	–	0.435	0.334	0.101
	cg04205769	1	DPH5	0.488	0.388	0.101
Hypermethylated	cg07179329	16	CDH13	0.552	0.764	0.213
	cg02228913	17	MAPT	0.789	0.920	0.130
	cg24801230	17	MAPT	0.701	0.823	0.121
	cg05280698	19	HKR1	0.372	0.485	0.113
	cg01911613	3	–	0.158	0.268	0.110
	cg22203628	11	–	0.456	0.563	0.107

Table 3
Ingenuity pathway analysis (IPA) of genes associated with significant CpG sites based on disease state: lupus and non-lupus.

Ingenuity Canonical Pathways	P-value
Interferon Signaling	9.12E-04
Sumoylation Pathway	7.08E-03
14-3-3-mediated Signaling	1.17E-02
Embryonic Stem Cell Differentiation into Cardiac Lineages	1.23E-02
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	1.26E-02
p70S6K Signaling	1.26E-02
Diphthamide Biosynthesis	1.48E-02
RAR Activation	2.51E-02
Role of Lipids/Lipid Rafts in the Pathogenesis of Influenza	2.82E-02
Huntington's Disease Signaling	3.80E-02
Sonic Hedgehog Signaling	3.80E-02

2.6. RNA extraction and qRT-PCR analysis

cDNA was processed from total RNA, which was extracted using Clontech Advantage® RT-for-PCR kits (Clontech, Mountain View, CA). The resulting cDNA was amplified using qRT-PCR multiplex assays (multiplex power mix solution, Bio-Rad Hercules, CA). The list of primers included *GAPDH, β-actin, MX1, IFI44L, IFIT1, IRF7, and PLSCR1* (Integrated DNA Technologies, Coralville, Iowa) (Supplemental Table 1). All samples were assayed in triplicate using a Bio-Rad CFX96 C1000 system (Bio-Rad Hercules, CA). Expression levels were normalized with the endogenous control genes *GAPDH* and *β-actin*. The resulting ΔCT values were analyzed using the linear model described for differential methylation analysis. Similarly, multiple comparisons were corrected adjusted using the "global" approach in *limma*. We evaluated the significance of three pairwise comparisons: 1) NL vs Lupus, 2) NL vs SLEDAI > 6, and 3) AA NL vs AA Lupus. For each pairwise comparison, fold-change was determined using $2^{-\Delta\Delta CT}$ calculations. All data analysis was performed in R.

3. Results

A description of the covariates for the lupus and NL donors used in this study is provided in Table 1. There was a significant difference in age between the lupus and NL samples in the methylation and qRT-PCR studies. However, the proportion of samples based on ethnicity was not significantly different in either study. For this study, the criteria for statistical significance of CpG sites were FDR < 0.05 and |beta difference| ≥ 0.1.

Table 4
Significant hypomethylated (FDR-adjusted p-values < 0.05, |mean methylation β change| ≥ 0.1) CpG sites in lupus and non-lupus patients based of SLEDAI score. Sites associated with interferon-related genes are highlighted.

		CpG Site	Chr	Gene	Methylation β value		Δβ
					Non-lupus	Lupus	
Hypomethylation	**Non-Lupus vs. SLEDAI ≤ 6**	cg23283667	8	GPIHBP1	0.858	0.683	0.175
		cg06963709	7	–	0.633	0.511	0.122
		cg08349108	11	–	0.428	0.321	0.107
		cg25880954	1	MGC12982;FOXD2	0.887	0.781	0.106
		cg17069914	3	–	0.435	0.329	0.106
		cg19757535	***4***	***IRF2***	***0.704***	***0.599***	***0.105***
	Non-Lupus vs. SLEDAI > 6	***cg22930808***	***3***	***PARP9;DTX3L***	***0.691***	***0.395***	***0.295***
		cg00959259	***3***	***PARP9;DTX3L***	***0.564***	***0.288***	***0.276***
		cg01079652	***1***	***IFI44***	***0.953***	***0.723***	***0.230***
		cg06981309	***3***	***PLSCR1***	***0.495***	***0.302***	***0.193***
		cg15065340	3	TNK2	0.659	0.512	0.147
		cg08926253	***11***	***IRF7***	***0.613***	***0.467***	***0.146***
		cg01521397	20	TAF4	0.498	0.356	0.141
		cg08265274	6	HLA-DRB5	0.779	0.639	0.139
		cg12162100	8	CPNE3	0.543	0.404	0.139
		cg10827754	2	–	0.531	0.392	0.139
		cg25199552	1	KDM1A	0.547	0.412	0.135
		cg23213327	***2***	***RSAD2***	***0.525***	***0.393***	***0.132***
		cg17301248	12	NAA25	0.441	0.311	0.130
		cg02353916	4	LOC285550	0.511	0.381	0.130
		cg16788865	12	–	0.436	0.307	0.129
		cg25814293	7	MAD1L1	0.683	0.555	0.128
		cg01054652	11	–	0.585	0.458	0.127
		cg12401842	12	CLEC4A	0.484	0.361	0.123
		cg12213414	2	LYG2	0.621	0.501	0.120
		cg20107506	4	–	0.513	0.394	0.119
		cg08110693	6	PXT1	0.565	0.448	0.117
		cg12354192	3	TNIK	0.623	0.507	0.116
		cg17980508	***1***	***IFI44L***	***0.292***	***0.180***	***0.112***
		cg24496021	11	FCHSD2	0.505	0.393	0.111
		cg02827175	7	MAD1L1	0.603	0.491	0.111
		cg18770635	22	–	0.711	0.600	0.111
		cg16261114	15	LOC145814	0.467	0.356	0.111
		cg04196862	17	HOXB5;LOC404266	0.494	0.383	0.111
		cg14272075	17	RNF126P1	0.495	0.386	0.109
		cg05310486	15	–	0.511	0.403	0.109
		cg02584498	2	GREB1	0.466	0.358	0.108
		cg09447675	12	–	0.513	0.405	0.108
		cg07772516	15	TMOD2	0.409	0.301	0.108
		cg11576590	1	–	0.498	0.391	0.107
		cg21964800	11	LGALS12	0.577	0.470	0.107
		cg01190666	20	PRIC285	0.570	0.464	0.106
		cg14784004	3	IRAK2	0.829	0.724	0.106
		cg23510089	4	–	0.758	0.653	0.105
		cg00211174	3	MCM2	0.442	0.338	0.104
		cg15361231	1	GLRX2	0.517	0.413	0.104
		cg06655349	19	S1PR2	0.457	0.353	0.104
		cg05624376	2	DHRS9	0.456	0.352	0.104
		cg18855195	22	RIBC2	0.483	0.379	0.104
		cg25448856	12	EEA1	0.524	0.420	0.104
		cg19935471	8	MATN2	0.566	0.463	0.103
		cg18830993	14	PELI2	0.511	0.408	0.103
		cg12405599	3	RPN1	0.465	0.362	0.103
		cg13982956	9	PBX3	0.308	0.205	0.103
		cg22446264	6	–	0.564	0.462	0.103
		cg08300570	7	CDK6;CDK6	0.473	0.370	0.103
		cg13573582	1	RNF220	0.491	0.389	0.102
		cg12379452	17	TRIM25	0.926	0.824	0.102
		cg26891210	1	CTSS	0.562	0.461	0.101
		cg07480762	3	PLCH1	0.405	0.304	0.101
		cg14220170	3	TRANK1	0.562	0.461	0.101
		cg06122230	20	ZNFX1	0.539	0.438	0.101
		cg16993108	7	MAD1L1	0.739	0.639	0.101
		cg21534299	1	ERI3	0.614	0.514	0.100
		cg01952989	7	MAD1L1	0.722	0.622	0.100
		cg12042714	15	–	0.357	0.257	0.100

3.1. Analysis of differential methylation between NL and lupus subjects

We identified 41 differentially methylated sites between NL and lupus subjects, which were associated with 30 genes. These CpG sites also demonstrated a significant difference in average methylation between lupus and NL subjects ($p = 0.0024$) (Fig. 1A). Of the 41

Table 5
Significant hypermethylated (FDR-adjusted p-values < 0.05, | mean methylation β change | ≥ 0.1) CpG sites in lupus and non-lupus patients based of SLEDAI score. Sites associated with interferon-related genes are highlighted.

		CpG Site	Chr	Gene	Methylation β value		Δβ
					Non-lupus	Lupus	
Hypermethylation	**Non-Lupus vs. SLEDAI > 6**	cg07179329	16	CDH13	0.552	0.812	0.260
		cg24801230	17	MAPT	0.701	0.853	0.151
		cg05280698	19	HKR1	0.372	0.507	0.135
		cg22203628	11	–	0.456	0.578	0.122
		cg24834889	19	HKR1	0.372	0.490	0.118
		cg00446123	20	LIME1	0.507	0.622	0.115
		cg01123250	2	UNC80	0.541	0.656	0.114
		cg23825480	22	MORC2	0.544	0.658	0.114
		cg03971555	1	PIK3CD	0.232	0.346	0.114
		cg21201401	20	LIME1	0.430	0.543	0.114
		cg14437551	6	LTA	0.581	0.693	0.111
		cg25042789	***6***	***TAP1***	***0.498***	***0.608***	***0.111***
		cg14137381	5	SLC9A3	0.323	0.432	0.109
		cg23917817	6	HLA-DPB2	0.549	0.658	0.109
		cg08169020	14	ZFP36L1	0.478	0.586	0.108
		cg23144994	7	FLJ40852	0.514	0.619	0.105
		cg18228076	17	MAPT	0.497	0.602	0.105
		cg23620279	10	RPS24	0.151	0.255	0.104
		cg17735539	10	ADARB2	0.460	0.562	0.101

differentially methylated sites, 85% (35 sites associated with 26 genes) were hypomethylated in lupus patients (Table 2). The significant hypomethylated genes included several interferon related genes: *MX1*, *IFI44L*, *PARP9*, *DTX3L*, *IFI44*, *PLSCR1*, *IRF7*, *IFIT1,* and *RSAD2* (Table 2). The two most hypomethylated sites in lupus compared to NL subjects were *MX1* (cg21549285; $\Delta\beta = 0.286$) and *IFI44L* (cg05696877; $\Delta\beta = 0.235$). Pathway analysis (missMethyl) of the 41 differentially methylated CpGs identified two significant KEGG pathways: influenza A (FDR = 4.15×10^{-3}) and herpes simplex infection (FDR = 4.15×10^{-3}). Interferon signaling (FDR = 9.12×10^{-4}) was identified as the top canonical pathway enriched in the 30 genes associated with the significant methylated sites (Table 3). The pathway network demonstrating how the differentially methylated genes are linked to lupus and interferon activity is presented in Fig. 1B.

3.2. Analysis of differential methylation between NL and lupus subjects based on SLEDAI score

Our analysis of NL vs lupus subjects with a SLEDAI score less than or equal to 6 (SLEDAI ≤ 6) and of NL vs lupus patients with a SLEDAI score greater than 6 (SLEDAI > 6) returned 6 and 79 significant CpG sites, respectively. These sites were associated with 4 and 62 genes, respectively. There was no significant difference in average methylation between the two groups for the NL-SLEDAI ≤ 6 comparison ($p = 0.3070$) (Fig. 1C), but there was a significant difference in average methylation between the NL and SLEDAI > 6 subjects ($p = 0.0043$) (Fig. 1D). The 6 significant sites for the NL-SLEDAI ≤ 6 comparison were all hypomethylated; only one was associated with an interferon related gene (cg19757535: *IRF2*, Table 4).

Of the 79 differentially methylated sites for the NL-SLEDAI > 6 comparison, 76% of the sites (60 sites associated with 44 genes) were hypomethylated (Table 4). The list of hypermethylated sites is presented in Table 5. The two most hypomethylated sites were both associated with PARP9; *DTX3L* (cg22930808, $\Delta\beta = 0.295$ *and* cg00959259, $\Delta\beta = 0.276$). Several of the significant sites were associated with interferon-related genes: *IFI44L*, *PARP9*, *DTX3L, IFI44*, *PLSCR1*, *IRF7*, *TAP1, TRIM25, CPNE3, MCM2, CDK6, RPN1,* and *RSAD2*. The pathway network illustrating how genes associated with significant CpGs for the NL-SLEDAI > 6 comparison are connected to lupus and interferon activity is shown in Fig. 1E. KEGG pathway analysis of the 79 differentially methylated sites identified 22 significant pathways, with influenza A as the top KEGG pathway (FDR = 3.92×10^{-3}, Supplemental Table 2). Phagosome maturation was identified as the top canonical pathway for the 62 associated genes ($p = 4.79 \times 10^{-4}$, Supplementary Table 2). Of the interferon related genes that were differentially methylated in NL vs lupus subjects, *IFI44L*, *PARP9*, *DTX3L*, *IFI44*, PLSCR1, *RSAD2*, and IRF7 were also differentially methylated in the NL-SLEDAI > 6 comparison.

3.3. Analysis of differential methylation between NL and lupus based on ethnicity (AA and EA)

There was not a significant difference in average methylation between EA lupus and EA NL subjects ($p = 0.9757$, Fig. 1F) or AA lupus and AA NL subjects ($p = 0.1852$, Fig. 1G). There were 23 differentially methylated sites in the EA NL-Lupus comparison (Tables 6 and 7). These CpGs were associated with 14 genes. Of the 23 sites, 53% (13 CpGs associated with genes) were hypomethylated (Table 6). None of the associated genes was involved in lupus or interferon activity. On the other hand, 58 CpGs (associated with 36 genes) were significantly methylated in the AA NL-Lupus comparison (Tables 6 and 7). Of the 58 sites, 64% (37 CpG sites associated with 21 genes) were hypomethylated in AA lupus patients (Table 6). The two most hypomethylated sites in AA NL-lupus comparison were cg21549285 (*MX1*, $\Delta\beta = 0.329$), and cg05696877 (*IFFI44L*, $\Delta\beta = 0.293$). Several of the significant sites were involved in interferon related activity: *MX1, IFIT1, IFI44L*, *PARP9*, *DTX3L, IFI44*, *PLSCR1*, *IRF7*, *DDX60, IFITM1, NLRC5, CMPK2, IFIT3, IFIT5, DOCK1* and *RSAD2*. Fig. 1H presents the pathway network demonstrating the relationship between these genes and lupus and interferon activity. KEGG pathway analysis of the 58 differential methylated sites identified three significant pathways, with influenza A as the top KEGG pathway (FDR-adjusted $p = 2.78 \times 10^{-2}$, Supplemental Table 3). Interferon signaling was identified as the top canonical pathway (*FDR* = 2.45×10^{-7}, Supplemental Table 3). Of the sites associated with interferon related genes for the NL-Lupus comparison, *MX1, IFIT1, IFI44L*, *PARP9*, *DTX3L, IFI44*, *PLSCR1*, *IRF7*, and *RSAD2* were also differentially methylated in the AA NL-Lupus comparison.

3.4. Correlation between methylation and SLEDAI score in interferon related genes

Based on the findings from differential methylation analysis, five interferon-related genes, *MX1*, *IFI44L*, *IFIT1*, *IRF7*, and *PLSCR1*, were selected for further correlation analysis. We computed Pearson's

Table 6
Significant hypomethylated (FDR-adjusted p-values < 0.05, |mean methylation β change| ≥ 0.1) CpG sites in lupus and non-lupus patients based of ethnicity (AA and EA). Sites associated with interferon-related genes are highlighted.

	CpG Site	Chr	Gene	Mean methylation β value		Δβ
				Non-lupus	Lupus	
European American (EA)	cg25880954	1	MGC12982;FOXD2	0.900	0.661	0.239
	cg13367219	12	–	0.504	0.347	0.157
	cg14062261	7	–	0.361	0.228	0.132
	cg08631502	16	–	0.268	0.141	0.128
	cg05313263	14	WDR25	0.428	0.313	0.115
	cg04663285	11	B4GALNT4	0.958	0.843	0.114
	cg14220170	3	TRANK1	0.563	0.451	0.112
	cg08302695	X	BMX	0.519	0.410	0.109
	cg10105794	1	–	0.673	0.564	0.109
	cg15056572	X	MIR505	0.599	0.492	0.107
	cg08487455	16	CNTNAP4	0.372	0.268	0.105
	cg26767960	2	SMARCAL1	0.878	0.775	0.103
	cg21931419	14	TMEM90A	0.503	0.402	0.101
African American (AA)	***cg21549285***	***21***	***MX1***	***0.760***	***0.431***	***0.329***
	cg05696877	***1***	***IFI44L***	***0.614***	***0.320***	***0.293***
	cg22930808	***3***	***PARP9;DTX3L***	***0.702***	***0.464***	***0.238***
	cg00959259	***3***	***PARP9;DTX3L***	***0.576***	***0.341***	***0.235***
	cg22862003	***21***	***MX1***	***0.653***	***0.434***	***0.219***
	cg05552874	***10***	***IFIT1***	***0.642***	***0.446***	***0.196***
	cg03607951	***1***	***IFI44L***	***0.504***	***0.313***	***0.192***
	cg06981309	***3***	***PLSCR1***	***0.501***	***0.328***	***0.174***
	cg16106427	10	STK32C	0.674	0.501	0.173
	cg06872964	***1***	***IFI44L***	***0.481***	***0.313***	***0.168***
	cg27298007	18	RAB31	0.499	0.335	0.163
	cg08122652	***3***	***PARP9;DTX3L***	***0.808***	***0.646***	***0.163***
	cg15954353	17	LOC728392	0.830	0.675	0.155
	cg09621330	16	–	0.704	0.549	0.155
	cg10176185	1	LDLRAP1	0.904	0.755	0.149
	cg26312951	***21***	***MX1***	***0.354***	***0.206***	***0.148***
	cg13304609	***1***	***IFI44L***	***0.888***	***0.751***	***0.137***
	cg07839457	***16***	***NLRC5***	***0.465***	***0.329***	***0.136***
	cg01028142	***2***	***CMPK2***	***0.893***	***0.757***	***0.136***
	cg06188083	***10***	***IFIT3***	***0.511***	***0.383***	***0.128***
	cg01079652	***1***	***IFI44***	***0.949***	***0.827***	***0.122***
	cg20107506	***4***	–	***0.525***	***0.404***	***0.121***
	cg23570810	***11***	***IFITM1***	***0.573***	***0.452***	***0.121***
	cg10549986	***2***	***RSAD2***	***0.221***	***0.101***	***0.120***
	cg10959651	***2***	***RSAD2***	***0.298***	***0.178***	***0.120***
	cg08926253	***11***	***IRF7***	***0.616***	***0.497***	***0.119***
	cg20098015	22	ODF3B	0.509	0.391	0.118
	cg09845944	10	JAKMIP3	0.985	0.868	0.117
	cg23213327	***2***	***RSAD2***	***0.546***	***0.430***	***0.116***
	cg15045292	17	LOC728392	0.783	0.670	0.113
	cg05883128	***4***	***DDX60***	***0.491***	***0.380***	***0.111***
	cg06963709	7	–	0.650	0.541	0.110
	cg06496041	21	ERG	0.803	0.694	0.109
	cg21534299	1	ERI3	0.606	0.499	0.107
	cg21984481	17	NPLOC4	0.854	0.748	0.106
	cg17984267	4	–	0.725	0.620	0.105
	cg13172359	***10***	***IFIT5***	***0.307***	***0.203***	***0.104***

correlation between methylation beta values and SLEDAI scores first for all lupus patients (Fig. 2A), and then for the stratified subgroups: 1) SLEDAI ≤ 6, 2) SLEDAI > 6, 3) AA, and 4) EA (Fig. 2B–E, respectively). Overall, when analyzing all the lupus patients, we found that the correlations were significant and moderately negative for all five interferon related genes. This was also true when analyzing the AA subgroups. In addition, we observed the same trend in the SLEDAI > 6 subgroup for *IFI44L*, *IRF7*, and *PLSCR1*, which were differentially methylated in the NL-SLEDAI > 6 comparison. The correlation for the SLEDAI ≤ 6 and EA subgroups was not significantly different from 0 for any of these genes.

3.5. qRT-PCR analysis of interferon related genes

The five interferon related genes, *MX1*, *IFI44L*, *IFIT1*, *IRF7*, and *PLSCR1,* were validated by qRT-PCR analysis for three pairwise comparisons: NL vs Lupus, NL vs SLEDAI > 6, and AA NL vs Lupus. *MX1* (FC = 2.1, global FDR = 0.0160), *IFI44L* (FC = 3.5, global FDR = 0.0227), *and IFIT1* (FC = 3.0, global FDR = 0.0386) showed significant differences in mRNA expression levels in NL and lupus patients (Fig. 3A). Although two of the five genes were not significantly different, all five interferon related genes demonstrated higher mRNA expression in lupus patients, which corresponded to the hypomethylation profiles observed in our methylation analysis. Although *IFI44L*, *IRF7*, and *PLSCR1* were differentially methylated in the NL-SLEDAI > 6 comparison, only IFI44L (FC = 5.0, global FDR = 0.0162) showed significant differences in mRNA expression levels (Fig. 3B). However, both IFI44L and PLSCR1 (FC = 2.0, global FDR = 0.1570) demonstrated an increase in mRNA expression in lupus patients with SLEDAI > 6 score compared to NL subjects (Fig. 3B), which corresponded to hypomethylation in SLEDAI > 6 lupus patients. Lastly, *MX1* (FC = 1.9, global FDR = 0.0229), *IFI44*L (FC = 4.2, global

Table 7
Significant hypermethylated (FDR-adjusted p-values < 0.05, |mean methylation β change| ≥ 0.1) CpG sites in lupus and non-lupus patients based of ethnicity (AA and EA). Sites associated with interferon-related genes are highlighted.

	CpG Site	Chr	Gene	Mean methylation β value		Δβ
				Non-lupus	Lupus	
European American (EA)	cg02228913	17	MAPT	0.628	0.878	0.250
	cg24801230	17	MAPT	0.527	0.764	0.237
	cg23762722	17	–	0.706	0.908	0.202
	cg21705961	17	MAPT	0.662	0.823	0.161
	cg14708411	5	SLC12A7	0.766	0.916	0.150
	cg18228076	17	MAPT	0.373	0.517	0.144
	cg13133387	17	–	0.724	0.845	0.121
	cg18591489	15	–	0.752	0.862	0.110
	cg11340603	12	SYT1	0.559	0.666	0.108
	cg20252997	1	LOC284661	0.492	0.598	0.105
African American (AA)	***cg06406458***	***10***	***DOCK1***	***0.706***	***0.909***	***0.203***
	cg06699216	8	CSGALNACT1	0.359	0.521	0.162
	cg19572051	***10***	***DOCK1***	***0.680***	***0.828***	***0.148***
	cg25471639	4	–	0.514	0.656	0.142
	cg26303281	1	S100A5	0.493	0.627	0.134
	cg10582608	6	–	0.320	0.451	0.131
	cg27662789	12	–	0.353	0.480	0.127
	cg19963856	17	SLFN12	0.106	0.230	0.124
	cg22452837	6	–	0.270	0.394	0.124
	cg18603538	7	LEP	0.538	0.659	0.121
	cg10753966	3	ERC2	0.552	0.669	0.117
	cg10960055	6	–	0.216	0.333	0.117
	cg10375710	17	DNAH17	0.743	0.858	0.116
	cg12595742	10	–	0.267	0.376	0.110
	cg08571475	6	–	0.855	0.964	0.110
	cg24476033	19	C3	0.217	0.325	0.108
	cg25852095	11	–	0.299	0.405	0.105
	cg27343917	2	FAM176A	0.531	0.636	0.105
	cg16124434	15	ITGA11	0.471	0.574	0.104
	cg06704969	3	C3orf21	0.799	0.903	0.104
	cg08289782	10	POLR3A	0.277	0.378	0.101

FDR = 0.0104), *IFIT1* (FC = 3.6, global FDR = 0.0002), and *PLSCR1* (FC = 2.4, global FDR = 0.0002) had significantly different mRNA expression in the AA NL-Lupus comparison (Fig. 3C). IRF7 was marginally non-significant (FC = 1.9, global FDR = 0.0543). All five interferon related genes demonstrated higher mRNA expression in AA lupus patients compared to AA NL patients (Fig. 3C), which corresponded to hypomethylation in AA lupus patients.

4. Discussion

SLE is a complex systemic autoimmune disease that affects many organs in the body. Emerging studies [24,25] have indicated that epigenetic mechanisms play a role in the pathogenesis of this disease, mainly due to its heterogeneous presentation and risk factors, such as environment, genetics, and an individual's immune response to diseases and infections.

4.1. Differential methylation between NL and lupus subjects

Our study confirmed similar findings from other studies [26,27] indicating that epigenetic deregulation may play an important role in lupus etiology. In addition to identifying epigenetic dysregulation in methylation profiles, mRNA expression, and signaling pathways (particularly the interferon signaling pathway), our study also identified differences in methylation profiles for African American and European American women with lupus. Previous published work from our laboratory [17] showed ethnic differences and global decreases in 5-methycytosine levels (hypomethylation) in lupus patients compared to age-matched healthy controls. Most epigenetic studies on lupus patients [7,28] did not explore the association between expression and disease activity scores. We noted differences in correlation between lupus patients with a SLEDAI score > 6 and those with score < 6, suggesting that epigenetics may play a role in the progression of this autoimmune disease.

Analysis of epigenome-wide DNA methylation studies from age-matched lupus and non-lupus African American and European American women revealed a number of interesting epigenetic signatures that have been shown to be significant in other GWAS lupus studies [7,10,29]. However, few studies have focused on ethnic differences in methylation profiles of specific genes or important regions within genes, in relation to disease activity scores in lupus patients. Our study examined DNA methylation levels for 485,000 ± CpG sites across the genome and identified 41 differentially methylated sites (associated with 30 genes) between lupus and controls subjects. Of the significantly methylated sites, 85% were hypomethylated. Like other GWAS studies [10,29], we also detected a significant “interferon signature” in Type-I interferon inducible genes, such as MX1, IFI44L, PARP9, DT3XL, IFIT1, IFI44, RSAD2, PLSCR1, DDX60, IFITM1, NLRC5, CMPK2, IFIT3, IFIT5, DOCK1 and IRF7.

Pathway network analysis of the 41 differentially methylated CpGs identified two significant KEGG pathways; influenza A and herpes simplex infection. The top significant canonical pathway was interferon signaling. Our study revealed a high expression of these genes. Elevated expression of interferon response genes has been shown to be associated with the risk of SLE [7,8,29]. Our data suggest that expression of these genes is through epigenetic changes such as hypomethylation.

Interferons are a family of cytokines that are key components of the innate immune systems. They serve as the first line of defense in an individual's fight against viral antigens and are involved in TH1-mediated immune response [30]. However, increased expression of IFNs and IFN-inducible genes has been implicated in the pathogenesis of SLE [7,8]. Viral DNA or RNA are typical activators of Type 1 IFN genes, that are known to promote B-cell activation and thus the production of autoantibody, which are hallmarks of lupus [31].

4.2. Differential methylation, differential expression, and ethnicity

MX1, (Myxovirus (influenza virus) resistance 1 gene) and IFI44L, (IFN-induced protein 44 gene) were associated with the two most hypomethylated CpG sites in lupus patients in this study, indicating an increased expression in MX1 and IFI44L. Increased levels of MX1 can induce an adaptive immune response that can lead to the breakdown of tolerance to self antigens [30]. MX1 expression has also been reported to play a major role in apoptosis and cytokine-mediated cell signaling, as well as lupus nephritis [32]. Furthermore, we observed significant differences in MX1 and IFI44L expression when comparing African American women with lupus to those without lupus. African American women have the more common and most severe forms of lupus nephritis, which often progresess to end-stage renal disease [33]. Hypomethylated sites were observed in the IFI44L gene in African American women, but not European American women. Studies [10,34] have suggested that IFI44L may be a biomarker for distinguishing patients with lupus from healthy individuals or those with other autoimmune diseases [34]; however, our study suggests it could be a particular biomarker in African American women with lupus. The function of IFI44L remains unknown.

In addition, we observed ethnic differences between lupus and non-lupus subjects in methylation profiles for CpG sites associated the phospholipid scramblase 1 (PLSCR1) and IFIT1 genes. PLSCR1 and IFIT1 were significant for African American subjects but not European American subjects. PLSCR1 regulates TLR-9 (Toll-like receptor 9) mediated Type 1 interferon production in plasmacytoid dendritic cells (pDCs) [35]. These findings confirms previous work in our laboratory [36] which demonstrated significant increases in TLR-9 in African

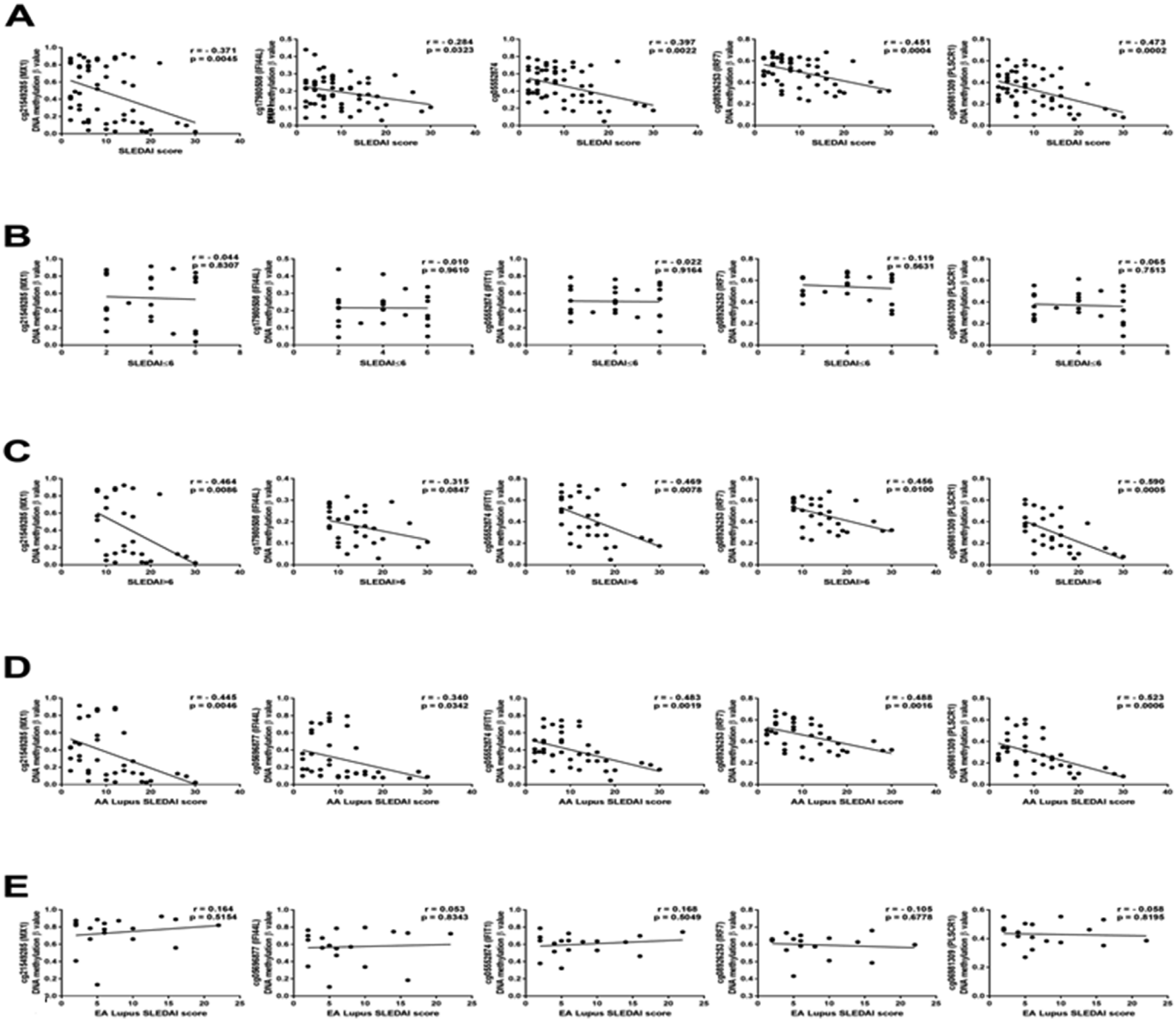

Fig. 2. Correlation between DNA methylation β values and SLEDAI scores for CpGs associated with the interferon-related genes, *MX1*, *IFI44L*, *IFIT1*, *IRF7*, and *PLSCR1*. Correlation between DNA methylation (β values) and SLEDAI score for (A) all lupus, (B) lupus-SLEDAI ≤ 6, (C) lupus- SLEDAI > 6, (D) AA lupus, and (E) EA lupus patients.

American women with lupus but not European American women with lupus. TLR-9 usually senses microbial or viral nucleic acids in the endosomes of pDCs and triggers MyD88-dependent Type I interferon responses [37]. Furthermore, it has been shown that PLSCR1, an IFN-inducible protein, interacts with TLR-9 and is involved in TLR-9 trafficking from the endoplasmic reticulum to the endosomes [37]. Others have shown increased expression of PLSCR1 in monocytes from patients with SLE and suggested that PLSCR1 may play an important role in Type I IFN responses during anti-viral innate immunity or in sensing self DNA/RNA in autoimmune diseases, such as lupus [38]. Canonical pathway analysis did identify genes associated with pattern recognition receptors in recognition of bacterial and viruses such as the TLRs. Studies, including those from our laboratory, have shown an enrichment of dysregulated genes in this receptor pathway [36,39]. Abnormal activation of TLRs is believed to breach immune tolerance and result in the production of cytokines, IFN-I, and autoantibodies [40].

Finally, our study has identified a number of hypomethylated interferon Type 1 genes in SLE patients which may serve as biomarkers for therapeutic development. A number of epigenetic drugs are currently in various stages of development in clinical trials for other diseases, but these data and other data suggest that these drugs may be potentially useful in lupus patients. However, further studies are needed to understand the ethnical differences in methylation profiles of interferon genes and genes identified in other pathways in this study.

5. Conclusions

We characterized the DNA methylome changes in lupus patients. Our data identified unique CpG sites that are epigenetically altered in lupus patients based on their ethnicity and SLEDAI score. It also provides evidence of DNA hypomethylation in interferon related genes in lupus. Here, we showed that when compared to control groups, AA lupus patients and lupus patients with SLEDAI > 6 have higher number of hypomethylated CpG sites, specifically in CpG sites associated with interferon-related genes, in comparison to EA lupus patients and lupus patients with SLEDAI ≤ 6, respectively. Thus, our findings support the

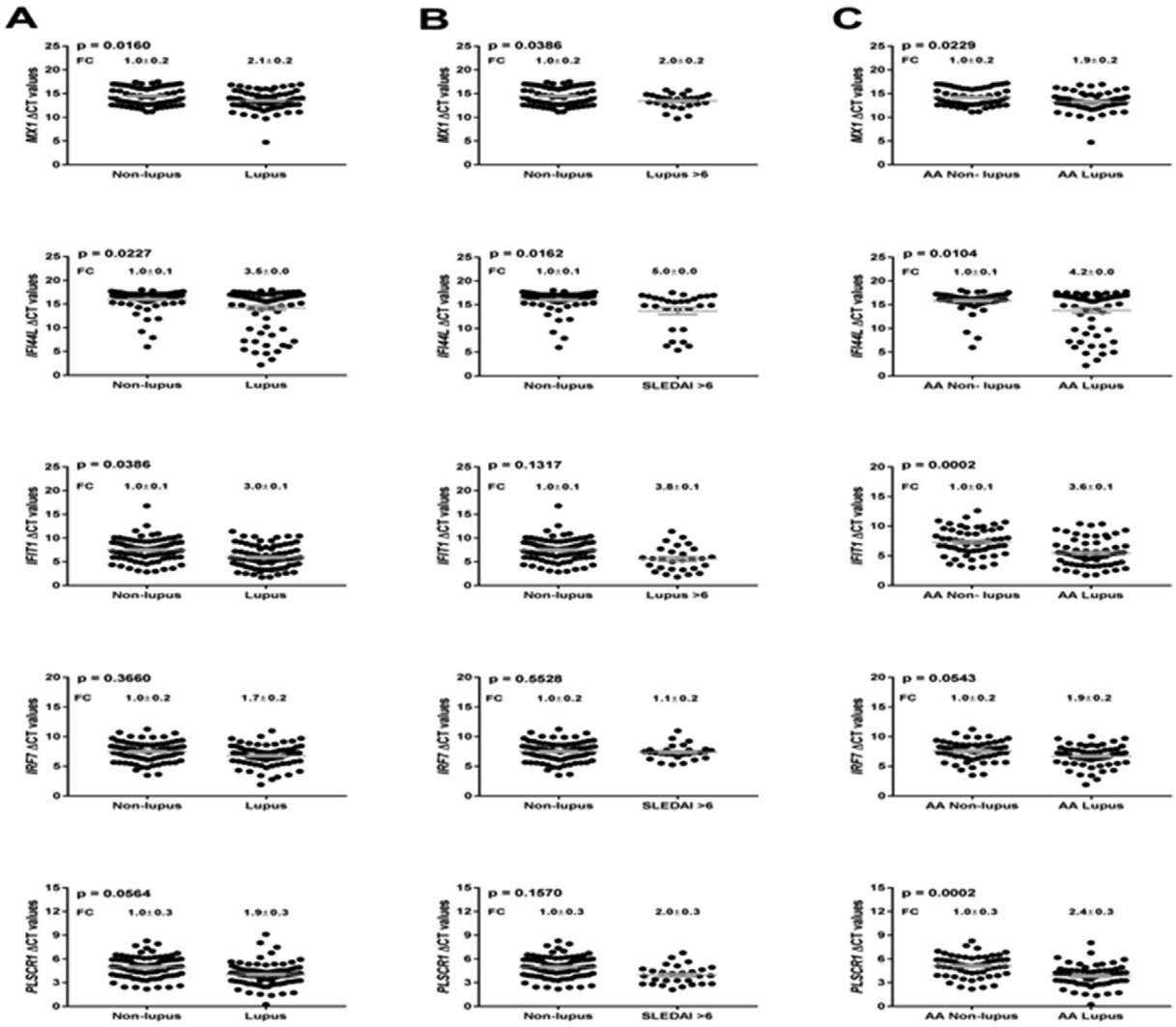

Fig. 3. QRT-PCR validation of the five interferon related genes, *MX1*, *IFI44L*, *IFIT1*, *IRF7*, and *PLSCR1*. Comparison of mRNA expression of the five validated genes, *MX1*, *IFI44L*, *IFIT1*, *IRF7*, and *PLSCR*1, between (A) NL and lupus, (B) NL and lupus-SLEDAI > 6, and (C) AA NL and AA lupus subjects using ΔCT values ($\Delta CT = CT_{gene} - CT_{endogenous\ control}$).

importance of the Type 1 interferon pathway in lupus pathogenesis and highlight differences in DNA methylation of lupus patients characterized by their SLEDAI score and ethnicity.

Conflicts of interest

None declared.

Disclaimer

The views presented in this report do not necessarily reflect those of the US Food and Drug Administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2018.09.007.

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