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The dynamics of methylation of CpG sites associated with SLE subtypes in a longitudinal cohort

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Abstract

Objective—Cross-sectional studies have revealed associations between DNA methylation and systemic lupus erythematosus (SLE) outcomes. To study the dynamics of DNA methylation, we examined participants from a SLE longitudinal cohort sampled at two time-points.

Methods—One hundred and one participants from the California Lupus Epidemiology Study were studied. DNA extracted from blood at cohort enrollment and after two years was analyzed using the Illumina EPIC BeadChip. Paired t-tests were utilized to identify changes at 256 CpG sites previously associated with SLE subtypes as well as genome-wide. Mixed linear models were developed to understand the relationship between DNA methylation and disease activity, medication use and sample cell proportions, adjusting for age, sex and genetic principal components.

Results—The majority of CpGs that were previously associated with SLE subtypes remained stable over two years (185 CpGs (72.3%), t-test FDR >0.05). Compared to background genome-wide methylation, there was an enrichment of SLE subtype associated CpGs that changed over time (27.7% vs 0.34%). Changes in cell proportion were associated with changes at 67 CpGs ($p<2.70E-05$) and 15 CpGs had at least one significant association with use of an immunosuppressive medication.

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Conclusion—In this longitudinal cohort of SLE, we identified a subset of SLE subtype associated CpGs that were stable over time and may be useful as biomarkers of disease subtypes. Another subset of SLE subtype-associated CpGs changed at a higher proportion compared to the genome-wide methylome. Additional studies are needed to understand the etiology and impact of these methylation changes in SLE-associated CpGs.

Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease that affects one in 600 women in the United States, and it is among the leading causes of death in young females, despite modern treatments.[1, 2] Prior studies suggest that epigenetics can inform SLE disease heterogeneity and pathophysiology. Epigenetics is the study of chromatin modifications, including DNA methylation, that regulate gene expression and cell differentiation.[3] Changes in methylation of CpG sites within interferon-responsive genes and regulatory regions of the genome and in different immune cell types are associated with SLE risk, disease activity, and specific organ manifestations such as lupus nephritis.[4–9] However, causality for these associations cannot be determined given the cross-sectional nature of previous studies. Furthermore, the stability of these changes and prognostic implications for long term outcomes remain unclear.

There are many challenges in treating SLE patients, and available biomarkers that can accurately predict clinical outcomes and response to treatment are lacking. DNA methylation derived from whole blood is an attractive biomarker, as samples can be easily obtained and do not require isolation of peripheral blood mononuclear cells or cell sorting. Thus, whole blood DNA methylation has the potential to be easily applied in clinical practice as a tool for precision medicine. Therefore, understanding the longitudinal stability and variability of the methylome in patients with SLE is fundamental to its utility as a biomarker.

In this work, we investigated the longitudinal trajectory of DNA methylation in whole blood among a diverse multi-ethnic cohort of patients with SLE followed over two years. We have previously performed an epigenome-wide association study (EWAS) of all participants of the California Lupus Epidemiologic Study (CLUES) at cohort enrollment.[10] We identified three patient subtypes at cohort enrollment based on American College of Rheumatology (ACR) classification criteria and subcriteria. We labeled them mild (M), severe 1 (S1) and severe 2 (S2), based on the pattern of autoantibodies and internal organ involvement, and identified 256 CpGs that were significantly associated with the subtypes, many of which mapped to the interferon pathway.

Here, we examined the dynamics of this previously described DNA methylation signature as well as the genome-wide longitudinal trajectory of the methylome in members of the CLUES cohort (n=101). We studied the impact of disease activity, medication use, cell proportions, genetic variation and self-report ethnicity and race on changes in methylation at CpG sites.

Patients and Methods

Subjects

The California Lupus Epidemiology Study (CLUES) is a multi-racial/ethnic cohort of individuals with physician confirmed SLE. This study was approved by the Institutional Review Board of the University of California, San Francisco. All participants signed a written informed consent to participate in the study. Participants were recruited from the California Lupus Surveillance Project, a population-based cohort of individuals with SLE living in San Francisco County from 2007 to 2009.[11] Additional participants residing in the geographic region were recruited through local academic and community rheumatology clinics and through existing local research cohorts. This study included a subset of 101 CLUES participants from the following self reported race/ethnicities: white (n=29), Black (n=13), Asian (n=34), Hispanic (n=22) and 3 other or unspecified. Clinical and demographic characteristics are shown in Table S1.

Study procedures involved an in-person research clinic visit every two years, including collection and review of medical records prior to the visit; a history and physical examination conducted by a physician specializing in lupus; collection of biospecimens, including peripheral blood for clinical and research purposes; and completion of a structured interview administered by an experienced research assistant. The average time between visits for the 101 participants was 2.3 years with a standard deviation of 0.3. All SLE diagnoses were confirmed by study physicians based upon one of the following definitions: (a) meeting ≥ 4 of the 11 American College of Rheumatology (ACR) revised criteria for the classification of SLE as defined in 1982 and updated in 1997, (b) meeting 3 of the 11 ACR criteria plus a documented rheumatologist's diagnosis of SLE, or (c) a rheumatologist confirmed diagnosis of lupus nephritis, defined as having evidence of lupus nephritis on kidney biopsy. Medication use at the time of blood draw was recorded. For data analyses, we grouped immunosuppresants into the following categories: biologics (belimumab, abatacept, rituximab), low dose prednisone (<10 mg), moderate or high dose prednisone (> 10 mg), antimalarials, calcineurin inhibitors, methotrexate and leflunomide, azathioprine, mycophenolate mofetil, and cyclophosphamide. Self-reported race and ethnicity information was collected from each study participant.

DNA methylation and quality control

Methylation of genomic DNA from whole blood was profiled using the Illumina MethylationEPIC BeadChip. This chip assesses the methylation level of ~850,000 CpGs in enhancer regions, gene bodies, promoters, and CpG islands. All subsequent processing was done using the R minfi package. Signal intensities were background subtracted using the minfi noob function and then quantile normalized.[12, 13] Sites with a poor detection rate (detection p value > 0.05) in more than 5% of the samples (1,746 CpG sites) were removed. Sites where probes were predicted to hybridize to multiple loci (44,097) and sites on non-autosomal chromosomes (19,627 CpG sites) were removed. We also excluded 91,799 CpGs that have been shown to perform poorly due to single nucleotide polymorphisms (SNPs) near probes in diverse populations.[14] Additionally, we removed 3,413 CpGs where the assay

control sample displayed a variance over 0.01 across the 9 plates. After implementing these quality control measures, 720,682 CpGs remained for analysis.

DNA genotyping

Genotyping of genomic DNA from blood was performed using the Affymetrix Axiom Genome-Wide LAT 1 Array. This genotyping array is composed of 817,810 SNP markers across the genome and was specifically designed to provide maximal coverage for diverse racial/ethnic populations, including West Africans, Europeans and Native Americans.[15] Samples were retained with Dish QC (DQC) ≥ 0.82. SNP genotypes were first filtered for high-quality cluster differentiation and 95% call rate within batches using SNPolisher. Additional quality control was performed using PLINK. SNPs having an overall call rate less than 95% or discordant calls in duplicate samples were dropped. Samples were dropped for unexpected duplicates in IBD (identity by descent) analysis or mismatched sex between genetics and self-report; for first-degree relatives, one sample was retained. All samples had at least 95% genotyping and no evidence of excess heterozygosity (maximum < 2.5*SD). We tested for Hardy-Weinberg equilibrium (HWE) and cross-batch association for batch effects using a subset of subjects that were of European ancestry and negative for double-stranded-DNA antibodies and renal disease to minimize genetic heterogeneity. SNPs were dropped if HWE $p < 1e\text{-}5$ or any cross-batch association $p < 5e\text{-}8$. Genetic principal components were calculated to account for population structure utilizing the PCAmixdata R package.

Genetic Ancestry

We performed an ADMIXTURE[16] analysis using genome-wide SNP data to estimate the percent contribution of each ancesteral population for each participant in the study. We first combined our sample data with 1000 Genomes genotype data and pruned SNPs for linkage disequilibrium according to software recommendations, removing each SNP with an R^2 value greater than 0.1 in a 50 SNP sliding window advanced by 10 SNPs each time. After pruning, 162,159 SNPs were used for global ancestry estimation. We then ran ADMIXTURE unsupervised assuming 5 subpopulations (K = 5, European/EUR, African/AFR, East Asian/EAS, South Asian/SAS, and Indigenous American/AMR). We then used known labels from 1000 Genomes to determine the ancestry of the estimated proportions for each of our subjects for downstream analysis.

Differential methylation analysis

Our analysis pipeline is shown in Figure 1. Samples from different time points were quantile normalized together. Principal component analysis (PCA) plots are shown between different time points, plates, and race/ethnicity (Fig S1–S3). Significance testing was performed utilizing M values, with conversion of effect sizes to Beta values for reporting. To adjust for plate, we utilized ComBat.[17] To account for cell proportion heterogeneity, we adjusted the Beta values with residuals for estimated cell proportions utilizing the reference-based Houseman algorithm.[18, 19] We initially performed a genome-wide paired t-test of the two time points and retained CpG sites that had a false discovery rate (FDR) p value of <0.05 and an absolute Beta value difference of more than 0.03, as a threshold for an effect size to be biologically meaningful.[20] We then took a closer look at the previous SLE-subtype

associated CpGs within the genome-wide results. We constructed repeated measures mixed linear models with DNA methylation as the outcome to investigate the effect of disease activity, dsDNA titer at the time of blood draw, lupus nephritis status and medication use adjusting for sex, age and genetic principal components (PC1-3). To examine the role of cell proportion heterogeneity, significant CpGs from the paired t-tests were re-analyzed without adjusting for cell proportions. Change in DNA methylation was modelled with change in each cell proportion as a predictor, adjusting for sex, age, and genetic principal components (PC1, PC2 and PC3). All association analyses were performed using R version 3.6 and Stata 13.1. Pathway analysis was performed with ToppFun.[21]

SLE-subtype associated CpGs enrichment analysis

Enrichment of SLE-subtype associated CpGs in CpGs with a significant change over time was determined via the following. Briefly, the methylation variance of the 256 CpGs associated with SLE subtypes at cohort enrollment was determined. Then, randomly selected 256 CpG sites with a similar methylation variance distribution than SLE subtype associated CpGs throughout the genome were tested to see if there was any difference in methylation at the two time points (paired t-test). We did this for a total of 100 random samples. Results were compared to CpGs associated with SLE subtypes.

Statistical methylation quantitative trait loci (meQTLs) analysis

We had previously reported on meQTL analysis findings on SLE-subtype associated CpGs at cohort enrollment.[10] Briefly, with this was performed by fitting a linear model adjusted for sex, age, cell proportions alcohol use, smoking status, the top three genetic principal components, and the top three medication principal components using the Matrix eQTL R package.[22] There are also larger established datasets of CpGs in healthy individuals with evidence of genetic control.[23] Combining our own findings and available resources, we identified a total of 39,899 CpGs with evidence of meQTL within the Illumina MethylationEPIC BeadChip that passed our quality control. A two sample test of proportions was utilized to see if the proportion of meQTLs in CpGs with a significant change over time was higher than the proportion of meQTLs in stable CpGs.

Results

The majority of the previously described CpGs associated with SLE subtypes remained stable over time.

In previous work, CLUES cohort participants were clustered into three subtypes according to ACR classification criteria at cohort enrollment. We identified 256 CpGs that were differentially methylated according to subtype.[10] For the current study, we observed the dynamics of our previous DNA methylation findings by comparing data collected at two time points. Of the 256 CpGs that were associated with disease subtypes, 184 CpGs (71.8%) showed stability between the two time points. Since we observed an enrichment of CpGs in IFN-responsive genes within the 256 CpGs, we investigated whether there was a difference in dynamics between CpGs of IFN responsive genes vs non-IFN responsive genes. We found that 53% of CpGs in IFN-responsive genes were stable vs 87% of CpGs in non-IFN responsive genes (chi square, p=1.4e-09), indicating that CpGs in IFN-responsive genes

are more susceptible to methylation change. Regarding CpG position relative to genes, 74 (39%) were in gene bodies, 50 (26.3%) in transcription start sites, and 30 (15.7%) in UTR regions. Table 1 highlights 20 CpGs with the most variance across clinical clusters[10] that did not change over time, and the full list of stable CpGs can be found in Table S2. These include CpGs in *TNK2, RABGAP1L, IRF7, IFI44L*, *TRIM22* and many IFN-responsive genes. DNA methylation within/near these genes has been implicated in SLE in previous studies, for example *TNK2* with renal disease in CD4+ naïve cells[4] and *RABGAP1L* with anti-dsDNA antibody production. Volcano plots of representative stable CpGs are shown in Figure S4.

A higher proportion of SLE-subtype associated CpGs changed over time compared to the genome-wide methylome.

Although the majority of SLE-subtype associated CpGs were stable, 71 (27.7%) had a significant change in methylation (FDR p<0.05, methylation change > 0.03) (Fig 2a). We also examined the dynamics of the methylome genome-wide. Paired t-test analysis revealed that the methylation level of 2,423 CpGs across the genome changed significantly over a period of two years (FDR < 0.05), which is 0.34% of the represented methylome (Fig 2b). We also filtered results using a minimum DNA methylation difference (absolute Beta value difference > 0.03) and observed 309 CpG sites that demonstrated the minimum difference and passed FDR (Fig 3, Table S3). These CpGs were distributed across the genome with top results within/near *IFI44L, IFIT1, LOC101927924*, and *MX1*. The top 25 results by smallest p-value are shown in Table 2. Pathway analysis of the genes containing these 309 sites identified the human immune response to tuberculosis and RIG-1-like receptor (RLR) pathways as the most significant pathways, however, multiple immune pathways were represented, including antigen processing, virus response, type II IFN signaling, cytotoxic T lymphocytes pathways, and taurine and hypotaurine metabolism (Table 3.) When we looked across S1, M1 and M2 clinical subtypes, no significant difference in methylation change within changing CpGs was identified between the three subtypes (ANOVA test, FDR >0.05).

In comparison to the genome-wide results, there was strong evidence for enrichment in SLE subtype associated CpGs that changed over time (27% vs 0.34%, p= 1.82e-175). These included CpGs in *IFI44L, MX1* and *RAPGAB1L*. Sixty-eight of these 71 CpGs had a decrease in methylation and only three had an increase in methylation at the second time point. An enrichment analysis was performed supporting this finding. In 63 times out of 100, no CpG showed a significant difference in methylation (paired t-test FDR > 0.05, methylation beta difference < 0.03). In 2 samples, 3 CpGs had a significant change (p<7e-08, methylation beta difference > 0.03), 5 samples had 5 significant CpGs and 30 samples 1 signficant CpG. The distribution of CpGs with a significant change in the enrichment analysis is shown in figure S5.

CpG sites associated with clinical outcomes

Although disease was stable or quiescent over time in most study participants, a small percentage had significant changes in their clinical manifestations, such as changing dsDNA titers or development of lupus nephritis. For these cases, we evaluated whether CpG sites that changed over time were associated with SLEDAI score, dsDNA antibody

positivity and/or lupus nephritis (Table S4). Overall, no CpGs met the threshold of significance (p<1.8E-05) but some evidence for association was observed for cg09858955 in *VRK2* (coefficient −1.2, p=0.001), cg09128104 in *RARA* (beta=0.69 p=0.00036), and cg21524061 in *TLR6* (beta=045 p=0.0005) with SLEDAI score. These genes are involved in granulopoiesis (*RARA*),[24] apoptosis (*VRK2*)[25] and immune activation (*TLR6*)[26]; all of which are pathways relevant to lupus disease pathogenesis. Top associations with dsDNA positivity were cg01971407 in *IFITM1* (beta=−0.30 p=0.0003), cg05070493 in *TRAF3* (beta=−0.06 p=0.0003) and 2 CpGs in *PARP9* (cg00959259, beta=−0.029 p=0.0003; cg08122652, beta=−0.11 p=0.0004). Similarly, TRAF3 (TNF receptor-associated factor 3) is known as a powerful negative regulator of B cell survival and activation,[27] *IFITM1* is an IFN-responsive gene, and *PARP9* is associated with macrophage activation.[28, 29]

Effect of medications on DNA methylation

Since medications such as prednisone and methotrexate can alter the methylome in immune cells, we examined whether the changes in methylation at CpG sites were associated with the use of particular medications in a repeated measures model, adjusting for age, sex, and genetic PCs. Fifteen CpGs out of the 309 (4.9%) had at least one significant association with use of an immunosuppressive medication (Table S4). Nine CpGs were correlated with prednisone, 5 CpGs with mycophenolate mofetil, and 2 CpGs with azathioprine. There were no significant associations with changes in biologics, inhibitors of purine and pyrimidine synthesis, calcineurin inhibitors or antimalarials.

Effect of cell proportions on DNA methylation

One of the limitations of utilizing whole blood DNA methylation measures in population-based studies is that differences in methylation might be due to differences in cell proportions between individuals at the time of blood draw or changes taking place in cell proportions between blood draws over time. As expected, paired comparisons between the 6 estimated cell types at the two time points revealed significant changes in proportions of monocytes, granulocytes and CD8+ cells (paired t-test p< 0.05). In studies of SLE, overadjustment of cell proportion differences may lead to incorrect conclusions, as changes in cell proportions may be relevant to disease pathogenesis. To address these issues, we initially looked at the adjusted matrix for cell proportions to see if there were any DNA methylation changes. Then, to examine the effects of changes in cell proportions on change in methylation we utilized the unadjusted matrix and longitudinal models incorporating the difference in DNA methylation as outcome and the difference in each cell type as a predictor, adjusting for age, sex, and genetic PCs. We observed that 67 CpGs of the initial 309 (21.6%) that changed over time had a significant correlation with changes in at least one cell type estimate (p<2.70E-05). Changes in DNA methylation correlated with changes in cell proportions at 64 CpGs for granulocyte estimates, 39 CpGs for CD4+ T cell estimates, 24 CpGs for CD8+ T cell estimates, 5 CpGs for monocyte estimates, 12 CpGs for B cell estimates and 6 CpGs for NK cell estimates. Figure S6 shows the effect sizes of changes in DNA methylation in relation to changes in cell proportions. Although the largest number of CpGs influenced by changes in cell proportions was for granulocytes, the largest effect sizes were observed for the NK cell estimates, for example, cg0571263 (Beta= −20.25 p=5.9e-08), *IFITM1* (cg09026253, Beta= −13.95, p=6.4e-07), and *RAB6B* (Beta=14.55

p=1.6e-05). *RAB6B* has been shown to be expressed in NK cells as well as MAIT T-cells.[30] Other interesting examples of correlations of change in DNA methylation with change in cell proportions were in genes known to be enriched in a particular immune cell type. For example, methylation at *RPS6KB1* correlates with B cell estimates (cg02095219, beta= 10.01, p= 4.839e-07), where RPS6KB1 expression is known to be enriched in naïve B cells and memory B cells.[31] Other examples include CD4+ T cell estimates with methylation in *B2M (cg03425812*, beta=−7.4524419 p= 2.489e-08) and *IFIT1M* (cg04582010, beta= −6.40 p= 4.540e-08), known to be expressed widely in multiple CD4+ T cell subsets.[30, 32] Methylation at *B36NT3* (cg16744531, beta= 5.50, p= .00001) was associated with CD8+ T cell estimates, shown to be expressed in memory CD8+ T cells; methylation of *TNFSF10* (cg10213935, beta= −6.633 p= .000012) was associated with monocyte estimates, where TNFSF10 expression is known to be enriched in intermediate, classical and non-classical monocytes. And finally, methylation at *IFITM1* (cg05552874, beta= −2.93 p= 2.136e-06) and *HDAC4* (cg27074582, beta= −1.52681 p= 1.794e-09) was associated with granulocyte estimates.

Effect of self-reported ethnicity/race, genetic ancestry and genetic variation on methylation changes over time.

There is substantial evidence that DNA methylation differs across ethnic groups. Some of these differences are due to genetic variation and some are not explained by genetics alone.[33, 34] To examine the effects of genetic ancestry as well as self-reported ethnicity/race, we constructed models with methylation difference over time as the outcome and self-reported ethnicity/race or genetic ancestry estimates as predictors, adjusting for age and sex. Nine models were generated: one each for self-reported Hispanic, Black, White or Asian and genetic ancestry estimates AFR, EAS, AMR, SAS, EUR). Results are shown in the supplementary dataset S5. No model had a significant p value for multiple hypothesis testing (p<of 3.2 E-05), however there are a few associations that are worth mentioning. The top methylation change association was at cg23876832 (no gene name associated with this methylation site) with SAS ancestry (p= 3.75E-05). When looking at results with a nominal p-value <0.05, we find methylation change associations in 30 CpGs with AFR ancestry, 5 CpGs with EUR ancestry, 12 with AMR ancestry, 13 CpGs with EAS ancestry, and 12 CpGs with SAS ancestry. There were few overlaps, with 8 CpGs having an association with 2 ancestries. When examining self-report ethnicity/race, the top methylation change association fell in cg00569896 (no gene name associated with this cite) with Black race (p= 1.68 E-04). Results with a nominal p value <0.05 showed methylation change associations in 23 CpGs with Black race, 19 CpGs with Asian race, 8 CpGs with Hispanic ethnicity and 2 CpGs with White race. Similarly, there were few overlaps, with 5 CpGs showing an association of p <0.05 in 2 ethnicity/racial groups. In terms of overlap of CpGs with p<0.05 between ancestry and self report ethnicity/race, we found 20 CpGs with Black race and AFR ancestry, 2 CpGs with Asian race and EAS ancestry, no overlap between Asian race and SAS ancestry, 2 CpGs with Hispanic ethnicity and AMR race, and no overlap between EUR ancestry and White race. These results suggest that both ancestry and self-report ethnicity/race may be influencing methylation change, and primarily at different sites. The only exception is the high concordance between CpGs associated with Black race and AFR ancestry.

To test if genetic variation was influencing change in methylation at specific sites, we looked at CpGs known to be under genetic control (meQTLs). Of the 309 CpGs that changed over time, there were 75 CpG with evidence of meQTL (24%). This is in comparison with 5.5% of meQTLs in stable CpGs genome-wide (n=39,824), suggesting an enrichment of meQTLs in CpGs with a significant change in methylation over time (two-sample test of proportions p=4.8e-47). Of the 72 SLE subtype associated CpGs that changed over time, 24 (33%) are meQTLs. This is slightly higher than the proportion of meqtls in SLEs subtype associated CpG that did not change over time, but not statistically significant (n=46, 25%, two-sample test of proportions p=0.179).

Discussion

In this study, we examined the dynamics of DNA methylation for CpGs previously associated with SLE subtypes in a longitudinal cohort of SLE patients. Overall, we observed that a large proportion of SLE-subtype associated CpGs did not show significant change over two years. However, a much higher proportion of SLE-subtype associated CpGs changed over time compared to the genome-wide methylome. Some of the methylation changes observered over 2 years in SLE-subtype associated CpGs were associated with changes in cell proportions (26%) and medication use (4.5%).

As the epigenome is not static, an important question related to EWAS is if associations would change over time. These results are encouraging, providing evidence that overall, the methylation status of the majority of CpGs that were previously associated with specific SLE subtypes remain unchanged over a 2-year period. Therefore, the blood methylome shows potential as a biomarker for disease subtypes. This is further supported by a recent longitudinal study examining DNA methylation in circulating granulocytes from SLE patients, where authors also observed significant stability of the methylome over a period of 4 years.[35] With this in mind, we also refined our previous EWAS findings at cohort enrollment, selecting CpGs that had the most robust methylation difference between SLE subtypes and did not change over time. These candidate CpGs could be further studied prospectively at disease onset to examine their prognostic role in predicting SLE subtypes as well as potential biomarkers for treatment response.

We observed a very small number of CpGs for which DNA methylation changed significantly over time. Interestingly, pathway analysis showed that most of these CpGs are involved in immune-related pathways such as intracellular viral sensing pathways, antigen processing, IFN response as well as metabolic pathways (taurine metabolism, diabetes type II) (Table 3). We attempted to identify the underlying factors driving DNA methylation change. Overall, these changes were not correlated with disease activity, anti dsDNA antibody titer or lupus nephritis, although most cases in our cohort had quiescent disease. Although most of SLE subtype-associated CpGs were stable, there was a striking distinction between the increased proportion of SLE-associated CpGs that changed over time compared to genome-wide CpGs. One potential explanation for the observed progressive hypomethylation at SLE subtype associated CpGs is that PBMCs in SLE patients experience persistent exposure to the cytokine milieu inherent in SLE, making immune-related CpGs in circulating PBMCs more susceptible to change. This is

consistent with most SLE EWAS that demonstrate hypomethylation of immune-related genes in comparison with healthy controls as well as for severe SLE phenotypes compared to more mild disease.[5, 8–10] These findings have been attributed to defects in the enzymes responsible for maintaince of DNA methylation (DNA methyltransferases) due to oxidative stress.[36] Another potential hypothesis is that passive demethylation, the progressive loss of methylation over time, could be accelerated in SLE. The premise that passive demethylation can occur at different rates in individuals is the basis of epigenetic clocks or biomarkers of aging. These can drastically differ from chronological age.[37] Whether accelerated passive demethylation, or epigenetic aging, occurs in SLE relevant genes and pathways is unknown, but should be examined in future longitudinal studies with longer time periods of observation.

Studies with large sample sizes (> 1000) have estimated that at least 10% and up to 45% of the methylome is influenced by nearby meQTLs.[38] When we tested to see if genetic variation was influencing change in methylation, we found that a higher proportion of CpGs that changed over time had an meQTL association in comparison with stable CpGs. The leading hypothesis to explain cis-meQTL effects is that SNPs in protein binding sites alter or disrupt the activity of sequence-specific binding proteins such as transcription factors of methyl binding proteins which could lead to change in methylation patterns of nearby CpGs.[39–41] Since transcription factor binding is dynamic, one could postulate that this effect could influence the variability of methylation in addition to methylation itself. Other longitudinal methylation studies will be needed to corroborate this observation.

As expected, when we examined our initial results in the unadjusted matrix for cell proportions, we found that a substantial proportion (26%) of changing CpGs were associated with at least one cell estimate. This is an important consideration for studies that use whole blood DNA methylation to study the epigenetic landscape in SLE. As cell proportions in peripheral blood are of biologic relevance to disease pathogenesis, we are faced with a conundrum: how to deal with a potential confounder that could itself be a disease outcome? For the current study, we addressed this by initially adjusting for cell proportions and then re-examining findings in an unadjusted matrix to assess cell proportion effects. Further study of analytic approaches to whole blood DNA methylation data is important given the low cost and feasibility of working with whole blood in comparison to sorted or single cells, particularly for population-based studies that seek to provide a useful genomic clinical tool for precision medicine.

Limitations of this study include a relatively small sample size, which could have reduced our ability to detect a larger number of CpG sites that varied over time or fully assess the association between self-reported ethnicity/race and genetic ancestry. In addition, the detection of methylation fluctuations associated with disease activity was not possible due to the clinical quiescence of most participants. We were underpowered to identify additional CpGs sites associated with medication use. Finally, an extended time interval of more than two years could have yielded different findings. However, there have been few studies that have re-examined cross-sectional DNA methylation associations in a longitudinal cohort. Our rigorous analytic pipeline addressed potential limitations of studying whole blood DNA methylation in longitudinal studies, including effects of changes in cell proportions.

In summary, we have characterized the DNA methylation dynamics of previously SLE associated CpGs in the well characterized CLUES longitudinal cohort. Among these SLE subtype-associated CpGs, we have identified CpGs that are stable over time. Given their associaton with SLE syubtypes, these CpGs should be evaluated further for their potential role as biomarkers of disease outcomes. Additional longitudinal studies may also reveal weather SLE and immune related CpGs have an accelerated passive demethylation in comparison to the genome-wide methylome. Further study of the methylome dynamics in SLE at the time of disease flare and remission may provide additional insight into epigenetic programs that may guide the development of precision medicine approaches for SLE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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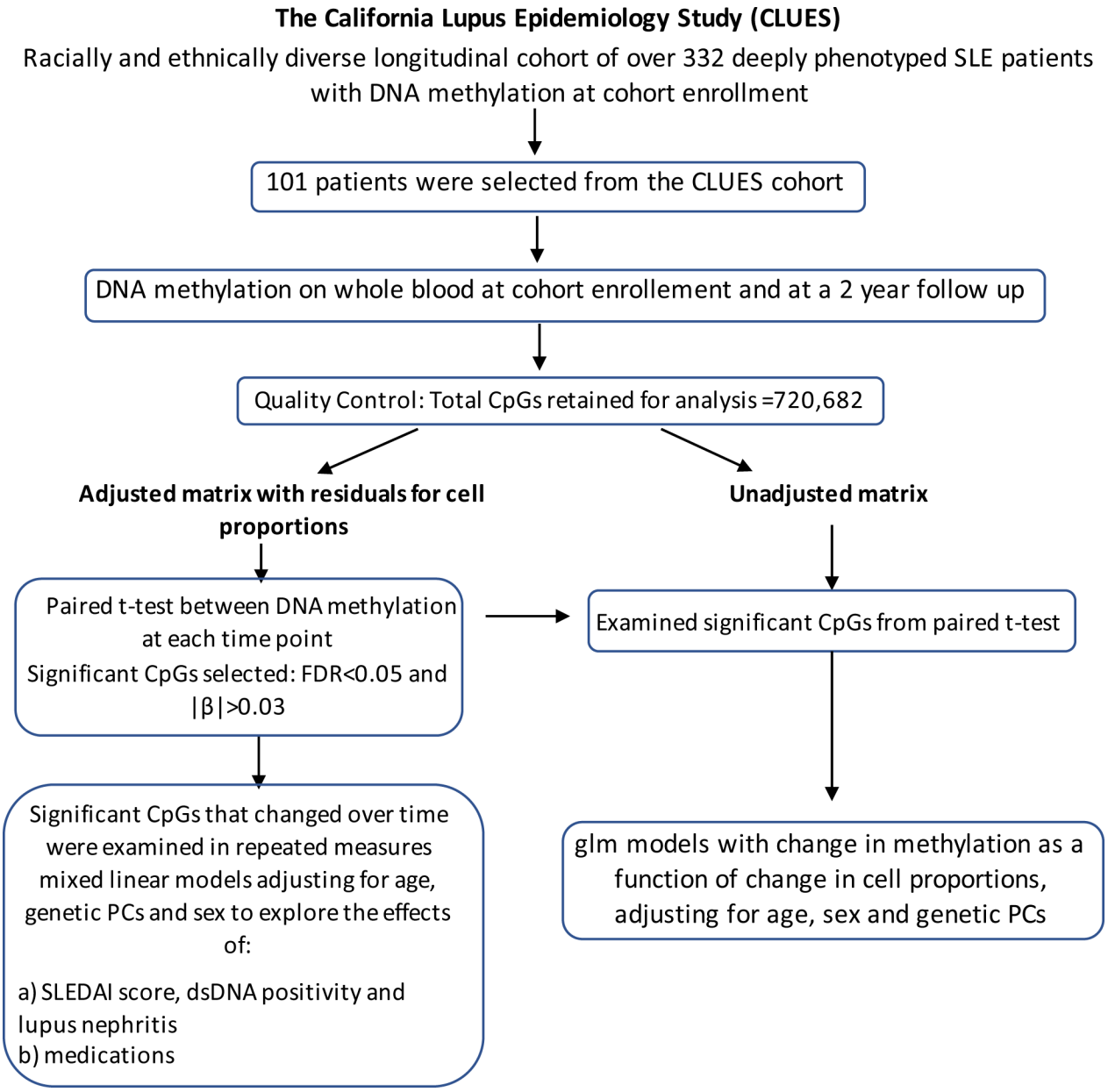

Fig 1. Differential DNA methylation analysis.

101 patients were selected for longitudinal DNA methylation analysis from the CLUES cohort (Table S1). Data were subjected to rigorous quality control procedures and processed for DNA methylation analysis (Fig S1). After removal of outliers/poor performing CpGs, we first performed a cell proportion adjusted analysis, and then reexamined our results in an unadjusted matrix to report on the effect of cell proportions. ComBat =R package, β= methylation beta value.

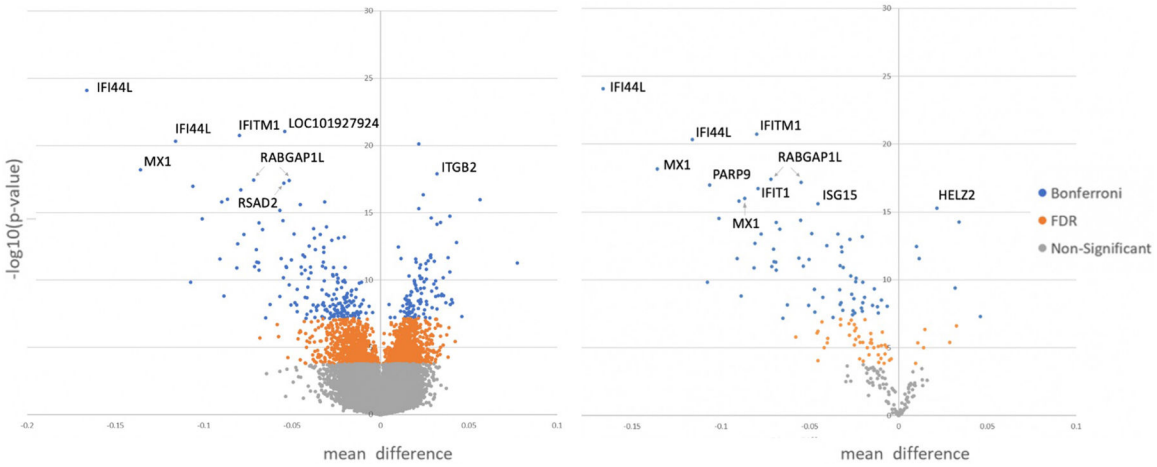

Fig 2. DNA Methylation dynamics in an SLE cohort.

Volcano plots of methylation differences between baseline and year 2. Mean methylation (Beta-values) are shown, adjusted for cell proportions and methylation plate. (A) SLE-subtype associated CpGs results, and (B) Genome-wide results. (significance line blue: p<1e-05, red: p<5e-8)

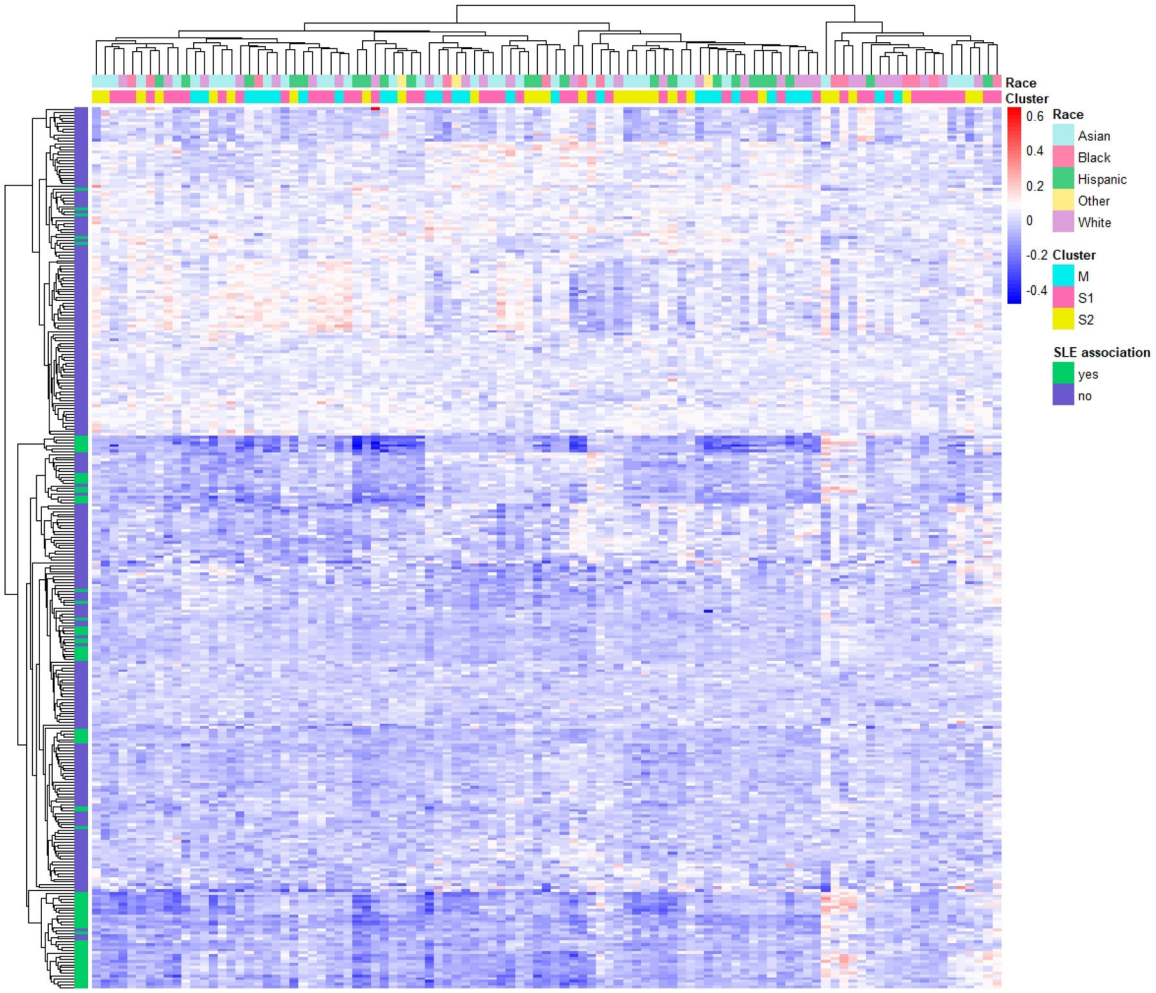

Fig 3. Heat map of CpG sites with a significant methylation change in a 2 year period.

We observed 309 CpGs with a DNA methylation difference (absolute Beta value difference > 0.03, FDR <0.05) in a 2 year time period. Each row represents a CpG and each column represents an SLE participant.

Table 1.

Selected candiate CpGs that were stable over time and had the most methylation difference between clinical sybtypes at cohort enrollment.

Mean methylation paired t-test, FDR > 0.05. Cohort partipiants were clusters based on ACR classification criteria and subcriteria using an unsupervised clustering approach.[10] Three clusters were identified. The clusters are labelled M (mild), S1 (severe 1) and S2 (severe 2).

cpg	Gene	CpG Position	Mean methylation beta values			Variance*	IFN-Alpha Responsive Gene	IFN Gamma Responsive Gene
			cluster M	cluster S1	cluster S2			
cg16987437	*SP100*	Body	0.623	0.536	0.481	0.0178	No	No
cg15065340	*TNK2*	5'UTR	0.623	0.555	0.499	0.0152	No	No
cg19188021	*ODF3B*	5'UTR	0.264	0.174	0.142	0.0142	No	No
cg17114584	*IRF7*	Body	0.513	0.451	0.399	0.0137	Yes	Yes
cg22012079	*IFI44L*	5'UTR	0.586	0.501	0.462	0.0126	Yes	Yes
cg12461141	*TRIM22*	TSS1500	0.493	0.423	0.380	0.0115	No	No
cg14333162	*RSAD2*	TSS1500	0.698	0.647	0.602	0.0092	Yes	Yes
cg26531432	*RABGAP1L*	5'UTR	0.698	0.634	0.605	0.0087	No	No
cg20343278	*PTPRM*	Body	0.323	0.361	0.304	0.0087	No	No
cg03540917	*SPINK2*	Body	0.599	0.627	0.669	0.0086	No	No
cg15378061	*NA*	NA	0.186	0.231	0.257	0.0084	No	No
cg15331332	*HLA-F*	Body	0.599	0.568	0.538	0.0081	No	No
cg00272009	*PARP14*	TSS1500	0.631	0.581	0.552	0.0080	Yes	Yes
cg25178683	*LGALS3BP*	TSS1500	0.554	0.509	0.470	0.0077	Yes	Yes
cg13045500	*NA*	NA	0.659	0.604	0.569	0.0072	No	No
cg06168856	*OAS1*	Body	0.630	0.598	0.575	0.0067	Yes	No
cg05167074	*SHKBP1*	Body	0.555	0.511	0.489	0.0067	No	No
cg06708931	*NA*	NA	0.906	0.867	0.828	0.0064	No	No
cg06650861	*DDX60*	5'UTR	0.868	0.830	0.792	0.0064	Yes	Yes
cg06376949	*IFIT5*	TSS1500	0.255	0.210	0.177	0.0063	No	No

*Anova F test

Table 2.

CpGs with significant change over a 2 year time period in the CLUES cohort.

The top 20 results are shown here. The full list of 309 CpGs can be seen in table S3.

		Mean methylation beta value				Paired t-test	
CpG	**Gene Name**	**Time 1**	**Time 2**	**Delta**	**fold change**	**p**	**FDR**
cg13452062	*IFI44L* *	0.31	0.14	0.17	−1.17	8E-25	5.97E-19
cg07929412	*LOC101927924*	0.75	0.70	0.05	−0.08	9E-22	3.33E-16
cg23570810	*IFITM1* *	0.49	0.41	0.08	−0.20	2E-21	4.32E-16
cg05696877	*IFI44L* *	0.31	0.19	0.12	−0.60	5E-21	8.45E-16
cg21549285	*MX1* *	0.40	0.26	0.14	−0.52	7E-19	7.94E-14
cg14628347	*ITGB2*	0.63	0.67	0.03	0.05	1E-18	1.33E-13
cg25984164	*RABGAP1L* *	0.71	0.63	0.07	−0.11	4E-18	3.29E-13
cg10549986	*RSAD2*	0.15	0.10	0.05	−0.51	4E-18	3.29E-13
cg09948374	*RABGAP1L* *	0.60	0.55	0.05	−0.10	6E-18	4.65E-13
cg07815522	*PARP9* *	0.45	0.34	0.11	−0.31	1E-17	6.88E-13
cg05552874	*IFIT1* *	0.40	0.32	0.08	−0.25	2E-17	1.14E-12
cg22862003	*MX1* *	0.41	0.33	0.09	−0.27	1E-16	5.06E-12
cg18467790	*RADIL*	0.52	0.58	0.06	0.10	1E-16	5.06E-12
cg16526047	*ISG15*	0.49	0.46	0.03	−0.07	2E-16	6.43E-12
cg24678928	*DDX60* *	0.70	0.61	0.09	−0.15	2E-16	6.43E-12
cg20062691	*ISG15* *	0.66	0.62	0.05	−0.07	3E-16	1.01E-11
cg07469075	*PAMR1*	0.58	0.52	0.06	−0.11	7E-16	2.44E-11
cg11317199	*TRIM14*	0.59	0.63	0.04	0.06	2E-15	5.99E-11
cg08565796	*HKR1*	0.32	0.35	0.03	0.08	2E-15	8.09E-11
cg12439472	*EPSTI1* *	0.31	0.21	0.10	−0.48	3E-15	8.94E-11
cg05883128	*DDX60* *	0.33	0.27	0.06	−0.20	4E-15	1.19E-10
cg13100600	*AGRN* *	0.51	0.54	0.03	0.06	5E-15	1.52E-10
cg07839457	*NLRC5* *	0.24	0.17	0.07	−0.40	6E-15	1.56E-10
cg25267487		0.67	0.71	0.03	0.05	7E-15	1.86E-10
cg13207212	*APBB2*	0.56	0.52	0.03	−0.06	1E-14	2.81E-10

* CpGs associated with SLE subtypes at cohort enrollment.

Table 3.

Pathway analysis of 309 CpG sites that showed significant methylation changes (FDR <0.05) and an absolute methylation Beta value difference of > 0.03 over 2 years. Pathway analysis was performed with ToppFun.[21]

ID	Name	Source	p value	FDR B&H	Genes from Input	Genes in Annotation
M39748	The human immune response to tuberculosis	MSigDB C2 BIOCARTA (v7.3)	2.82E-08	1.70E-05	6	23
M39583	Novel intracellular components of RIG-I-like receptor (RLR) pathway	MSigDB C2 BIOCARTA (v7.3)	1.20E-05	3.60E-03	6	61
M1462	CTL mediated immune response against target cells	MSigDB C2 BIOCARTA (v7.3)	1.59E-04	0.023	3	13
M39909	Host-pathogen interaction of human corona viruses - Interferon induction	MSigDB C2 BIOCARTA (v7.3)	1.64E-04	0.023	4	33
M22023	Antigen Processing and Presentation	MSigDB C2 BIOCARTA (v7.3)	2.01E-04	0.023	3	14
M39363	Type II interferon signaling (IFNG)	MSigDB C2 BIOCARTA (v7.3)	2.58E-04	0.023	4	37
M40067	SARS-CoV-2 Innate Immunity Evasion and Cell-specific immune response	MSigDB C2 BIOCARTA (v7.3)	2.68E-04	0.023	5	68
M15913	RIG-I-like receptor signaling pathway	MSigDB C2 BIOCARTA (v7.3)	3.28E-04	0.024	5	71
M39837	Cytosolic DNA-sensing pathway	MSigDB C2 BIOCARTA (v7.3)	3.98E-04	0.025	5	74
MAP00430	MAP00430 Taurine and hypotaurine metabolism	GenMAPP	4.22E-04	0.025	2	4
M19708	Type II diabetes mellitus	MSigDB C2 BIOCARTA (v7.3)	6.53E-04	0.036	4	47
M39543	Structural Pathway of Interleukin 1 (IL-1)	MSigDB C2 BIOCARTA (v7.3)	8.27E-04	0.042	4	50