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DNA methylation and childhood asthma in the inner city

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Abstract

Background—Epigenetic marks are heritable, influenced by the environment, direct the maturation of T lymphocytes, and in mice enhance the development of allergic airway disease. Thus it is important to define epigenetic alterations in asthmatic populations.

Objective—We hypothesize that epigenetic alterations in circulating PBMCs are associated with allergic asthma.

Methods—We compared DNA methylation patterns and gene expression in inner-city children with persistent atopic asthma versus healthy control subjects by using DNA and RNA from PBMCs. Results were validated in an independent population of asthmatic patients.

Results—Comparing asthmatic patients (n = 97) with control subjects (n = 97), we identified 81 regions that were differentially methylated. Several immune genes were hypomethylated in asthma, including *IL13*, *RUNX3*, and specific genes relevant to T lymphocytes (*TIGIT*). Among asthmatic patients, 11 differentially methylated regions were associated with higher serum IgE concentrations, and 16 were associated with percent predicted FEV₁. Hypomethylated and hypermethylated regions were associated with increased and decreased gene expression, respectively ($P < .6 \times 10^{-11}$ for asthma and $P < .01$ for IgE). We further explored the relationship between DNA methylation and gene expression using an integrative analysis and identified additional candidates relevant to asthma (*IL4* and *ST2*). Methylation marks involved in T-cell maturation (*RUNX3*), T_H2 immunity (*IL4*), and oxidative stress (catalase) were validated in an independent asthmatic cohort of children living in the inner city.

Conclusions—Our results demonstrate that DNA methylation marks in specific gene loci are associated with asthma and suggest that epigenetic changes might play a role in establishing the immune phenotype associated with asthma.

Keywords

DNA methylation; atopic asthma; epigenetics; T_H2 immunity; inner city

Asthma can be inherited and is affected by environmental exposures. To date, genome-wide linkage and association studies have identified more than 100 asthma-associated gene variants.^{1,2} However, sequence variants explain less than 10% of the risk of having asthma³, and several studies have shown that the risk of transmission of atopic disease from an affected mother is approximately 4 times higher than that from an affected father.⁴ Because epigenetic marks are also heritable and can account for parent-of-origin patterns of inheritance⁵, it is logical to speculate that epigenetic marks play a role in the transmission of asthma.⁶ Furthermore, although it is well established that asthma risk and severity are affected by specific environmental exposures, the epigenome can be altered by many of these environmental exposures, and these environmentally induced epigenomic changes often lead to rapid and persistent changes in gene expression.⁷ For example, *in utero* exposure to tobacco smoke is associated with childhood asthma, and this exposure can

modify gene expression through DNA methylation.^{8,9} In mice we have demonstrated that *in utero* supplementation with methyl donors alters locus-specific DNA methylation and predisposes mice to allergic airway disease by directing the differentiation of T lymphocytes, skewing toward a T_H2 phenotype.¹⁰ Importantly, epigenetic mechanisms have been shown to specifically affect the expression of transcription factors involved in the development of mature T lymphocytes (T_H1, T_H2, and regulatory T cells),^{11–13} providing a potential mechanism that links heritability, the environment, immune biology, and asthma.^{14,15} In candidate gene studies DNA methylation has been shown to be related to childhood asthma in peripheral blood cells,^{16,17} buccal cells¹⁸, or nasal epithelia.¹⁹ Thus epigenetic marks represent logical biological changes to pursue when considering the cause and pathogenesis of asthma.

Because black children and families living in poverty are at particularly high risk of asthma,²⁰ we have investigated the relationship between asthma and DNA methylation in African American children residing in the inner city. We hypothesize that epigenetic marks in circulating PBMCs are associated with allergic asthma.

METHODS

Study population

Our study population consisted of inner-city children aged 6 to 12 years with atopy and persistent asthma (cases, n = 97) and without atopy or asthma (healthy control subjects, n = 97) recruited by 6 sites of the Inner-City Asthma Consortium from census tracts that contain at least 20% of households living at less than the US government–defined poverty level.²¹ All subjects reported being African American, Hispanic with Dominican/Haitian background, or both. The validation population consisted of 101 African Americans between 6 and 12 years of age with atopic asthma collected by the Inner-City Asthma Consortium independent of the primary study population. Please refer to the Methods section in this article's Online Repository at www.jacionline.org for more information on selection of the study population.

DNA methylation and gene expression data collection

DNA methylation in PBMCs was measured on Illumina's Infinium Human Methylation 450k BeadChip (Illumina, San Diego, Calif) and validated by using pyrosequencing with custom-designed primers (see Table E1 in this article's Online Repository at www.jacionline.org). Gene expression was assessed on Nimblegen Human Gene Expression arrays (12x135k). Please refer to the Methods section in this article's Online Repository at www.jacionline.org for details of the protocols used for data collection.

Data quality controls

We first examined the quality control figures created by using the minfi R package for the 450k data.²² We observed a strong bimodal distribution of methylation values in the 450k data, as previously observed.²³ Further data quality for Illumina 450k and expression arrays was assessed by using principal components analysis (PCA). The principal components were examined for correlation with all clinical/demographic and laboratory data to identify

observable batch effects or covariates that explained variation (see Table E2 in this article's Online Repository at www.jacionline.org).²⁴ As a result of PCA, we removed one outlier sample from the 450k methylation data, whereas no outlier samples were identified in expression arrays. Although no laboratory variables were significantly correlated with principal components in the methylation data set, we included 4 laboratory variables in the expression analysis (labeling, date and concentration of whole transcriptome–amplified RNA, and hybridization date) because of their significant correlation with principal components (PCs). Of all demographic and clinical data, age and sex are strongly associated with the top PCs in both methylation and expression data sets. Although race/ethnicity is not associated with the top PCs, we included these covariates in the statistical model because of their known associations with DNA methylation.²⁵

In addition to PCA, probabilistic estimation of expression residuals (PEER) factors²⁶ were estimated to account for unknown batch effects. The number of PEER factors included in downstream analyses was 1 for 450k and 5 for gene expression. The number of PEER factors was based on the inflection point seen on visual inspection of the weighting of the inferred factors.²⁶

Overview of statistical analyses

The goal of our analyses was to determine whether DNA methylation and gene expression changes are associated with asthma, IgE levels, and percent predicted FEV₁. Cases and control subjects were used to determine whether methylation marks were associated with asthma, whereas the other 2 outcomes (IgE level and FEV₁) were analyzed only within asthmatic patients. We first identified differentially methylated regions (DMRs) associated with atopic asthma. For asthma-associated DMRs, we explored whether the DMRs were influenced by the cell composition of PBMCs. Next, we explored the relationship between PBMC gene expression and asthma, IgE levels, and percent predicted FEV₁ using the same approach outlined above. Lastly, we investigated the relationship of DNA methylation to gene expression in PBMCs. DNA methylation–gene expression relationships were examined by using (1) plots of gene expression versus DMRs and analysis of enrichment for inverse correlations of DMRs and gene expression and (2) integrated analysis of single-probe DNA methylation and gene expression. The second approach focused on single methylation probes as opposed to DMRs because of the limitation of statistical methodologies; all available methods for integrative analysis are limited to analysis of 1 methylation and 1 expression probe in statistical models.

Our rationale for inclusion of DMRs in this analysis is 4-fold: (1) identification of regions as opposed to single CpGs is conceptually consistent with what is known about DNA methylation patterns in the human genome²⁷; (2) DMRs increase power to detect associations^{28,29}; (3) DMRs allow for analysis of all probes on the array, which, in turn, facilitates finding regions of interest; and (4) DMR analysis has been used successfully in patients with other diseases.^{30–32} The alternative to DMR analysis is to remove between 20,000 and 140,000 probes from the analysis depending on the required frequency and distance of a variant in the African American population from the CpG motif. It is known that single nucleotide polymorphisms contained in the Infinium probes can affect binding

and result in spurious methylation measurements³³; however, our combined P value method³⁴ requires 2 or more adjacent probes and, as such, minimized this effect in the methylation analysis.

Statistical analyses of DNA methylation data

Data from the methylation array were normalized with the SWAN method³⁵ contained in the R package minfi,³⁶ and the normalized M -values were used in all downstream analyses. β Values (which range from 0–1) of all of the probes within each DMR are plotted in Figs 1, A, and 2, whereas average β values across the DMR are reported in Fig 3, A and B, and the supplemental tables. β Values are biologically intuitive interpretations of percentage methylation changes on the 0- to 1-point scale corresponding to 0% to 100% methylation.

We performed methylation array analysis in 3 steps. In brief, these are (1) infer PEER factors, (2) fit linear models with limma,³⁷ and use comb-p³⁴ to identify DMRs from the P values reported by limma. Limma was used to fit linear models because it allows us to use the moderated t statistic, which has been shown to be more powerful in microarray experiments than the standard t statistic. Our models included a clinical covariate of interest (asthma, FEV₁ asthma subtype, or IgE level), demographic covariates that are known to affect DNA methylation (age, sex, and race/ethnicity), and 1 PEER factor to account for unobserved batch effects (with details of statistical models included in Table E3 in this article's Online Repository at www.jacionline.org):

$$\text{Methylation} \sim \text{Asthma} + \text{Age} + \text{Sex} + \text{Race/ethnicity} + \text{Peer}(1).$$

$$\text{Methylation} \sim \ln(\text{IgE}) + \text{Age} + \text{Sex} + \text{Race/ethnicity} + \text{Peer}(1).$$

$$\text{Methylation} \sim \text{FEV}_1 (\% \text{ predicted}) + \text{Age} + \text{Sex} + \text{Race/ethnicity} + \text{Peer}(1).$$

In these models *Peer(1)* refers to the first PEER component. Cases and control subjects were used in the first model, whereas cases only were used in the second and third models. DMRs were identified from linear model P values by using comb-p,³⁴ with a window size of 300 bases and containing a minimum of 2 probes.

The identification of DMRs is described in detail in Pedersen et al³⁴ and outlined in the diagram in Fig E1 in this article's Online Repository at www.jacionline.org. Comb-p takes as input unadjusted P values for each probe on the array from limma and calculates an adjusted P value for each probe that accounts for the local correlation and the P values of neighboring probes within 300 bases. Probe-level P values are then adjusted by using the Benjamini-Hochberg methodology,³⁸ resulting in multiple testing-corrected P values for individual probes that are influenced by their neighboring CpG motifs. Next, comb-p finds regions or DMRs and calculates P values for the regions based on correlation (SLK P value).³⁹ Finally, the region P values are adjusted for multiple testing by using the Sidak correction⁴⁰ based on the size of the region and number of possible regions of that size, such that larger regions undergo less stringent correction because there are fewer possible large regions (SLK-Sidak P value). DMRs were annotated with the CruzDB tool.⁴¹

For validation, we analyzed pyrosequencing data from the discovery and validation cohorts using the 1-sided t test for cases versus control subjects and considered DMRs validated if they had P values of less than .05.

Estimate of cell proportions from DNA methylation data

We also analyzed Illumina 450k methylation data to estimate proportions of different mononuclear cell populations and contaminating granulocytes.⁴² Using our methylation β values and the 500 informative probes reported by Houseman et al,⁴² we estimated the coefficients for the 6 cell types: 5 monocyte cell types (B lymphocytes, CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, monocytes, and natural killer [NK] cells) and granulocyte contamination. We then used penalized regression to regress out the cell types and obtain a β value that represents the average cell. This process is encapsulated by the single R function `adjust.beta` (<https://gist.github.com/brentp/5058805#file-houseman-r>). We then logit transformed the adjusted β value to determine the M value and ran the model to determine which of the methylation differences were independent of cell type differences.

Statistical analyses of gene expression data

Intensity data from the Nimblegen DEVA software were \log_2 transformed and normalized by using RMA with the oligo R package.⁴³ The R package `limma`³⁷ was used to fit linear models for expression data, and P values were based on the moderated t statistic. For expression analysis, we used a model that incorporates clinical covariate of interest (asthma, \ln [IgE], or percent predicted FEV₁), demographic covariates that are known to affect expression (age, sex, and race/ethnicity), observable batch effects from PCA (labeling, hybridization date, WTA date, and WTA RNA concentration), and 5 PEER factors. The models for expression are presented in Table E3.

Correlation analysis of DNA methylation and gene expression

To understand the relationship of DMRs with gene expression changes, we considered inversely correlated (canonical) versus directly correlated pairs, limiting the analysis to genes within 3 kb of a DMR. We calculated the enrichment of inversely correlated pairs in relation to all pairs using the binomial test.

Integrated analysis of DNA methylation and expression

To integrate the expression and methylation data, we used LCMix, which was shown to be effective at identifying target genes in real and simulated data sets by combining information from disparate data sets.⁴⁴ LCMix requires a 1:1 relation between the input data sets, and therefore we limited our analysis to expression probes that had a methylation probe upstream within 3 kb of the transcription start site (23,848 genes). We then determined the number of components for the marginal fits to methylation and expression using integrated classification likelihood-Bayesian information criterion,⁴⁵ as suggested in Dvorkin et al.⁴⁴ Using those component numbers, we then performed joint fit using the chained topology (methylation and expression), 2 hidden components, and the Pearson type VII family.

Pathway and network analysis

We used Ingenuity Pathway Analysis⁴⁶ to identify protein-protein networks and enriched biofunctions in the data sets (using the Fisher exact test).

RESULTS

The study groups (97 healthy control subjects and 97 patients with atopic asthma) were selected from a larger population of potential study subjects who were specifically screened for this project (see Table E4 in this article's Online Repository at www.jacionline.org). When compared with control subjects, asthmatic children were more often male, more often exposed to damp home conditions, and less often exposed to gas stoves (Table I). Per study design, all asthmatic patients were atopic and had significant airflow limitation, as indicated by their total IgE levels and spirometric parameters (Table I).

Comparing asthmatic patients with control subjects and adjusting for age, sex, race/ethnicity, and unknown batch effects (the statistical model is shown in Table E3),³⁴ we identified 81 DMRs (Fig 1, A). Seventy-three DMRs were hypomethylated in asthmatic patients compared with control subjects, and 8 were hypermethylated (Table II). Several genes involved in T-lymphocyte biology were hypomethylated in asthmatic patients, including *IL13*, runt-related transcription factor 3 (*RUNX3*), and specific genes involved in the maturation and function of NK cells (*KIR2DL4*, *KIR2DL3*, *KIR3DL1*, and *KLRD1*) and T lymphocytes (*TIGIT*). Many of the genes associated with the 81 DMRs are components of regulatory networks that have been traditionally associated with asthma (Fig 1, B, and Table II, with remaining DMR regulatory networks presented in Fig E2 in this article's Online Repository at www.jacionline.org). The most DMRs are near genes that have not been previously implicated in asthma but are plausible asthma candidates, including alkaline phosphatase (*ALPL*), a gene with a role in modulating host-bacterial interactions by dephosphorylating LPS,⁴⁷ and Kruppel-like factor 6 (*KLF6*), a transcriptional activator with a recently established role in activation of inducible nitric oxide synthase 1 during influenza A virus infection⁴⁸ and activation of TGF- β during human respiratory syncytial virus infection.⁴⁹

To address the potential confounding effect of differential PBMC cell content, we used a regression calibration based on peripheral blood leukocyte cell-specific methylation to estimate the specific monocyte cell type and granulocyte contamination for each sample.⁴² Significant differences ($P = .004$) were observed between asthmatic and nonasthmatic groups only for the concentration of NK cells (see Table E5 in this article's Online Repository at www.jacionline.org). Fifty-five of the 81 DMRs remained significant after adjustment for cell mixture ($P < .05$), and an additional 8 approached statistical significance ($P < .1$, see Table E6 in this article's Online Repository at www.jacionline.org). As expected, the DMRs associated with NK cells (*KIR2DL4*, *KIR2DL3*, *KIR3DL1*, and *KLRD1*) were no longer significant after adjustment for differences in mononuclear cell populations, whereas *RUNX3* ($P = .04$), *IL13* ($P = 2.8 \times 10^{-5}$), and many other genes remained significant (see Table E6). Disease and biofunction enrichment analysis of the 55 DMRs identified organismal development, oversecretion of mucus and inflammatory/respiratory disease, and airway hyperresponsiveness among the most significantly enriched categories ($P < .039$,

Benjamini-Hochberg multiple comparison corrected; see Table E7 in this article's Online Repository at www.jacionline.org).

To determine whether specific measures of allergy or asthma are associated with DNA methylation, among asthmatic patients, we investigated the relationship between methylation marks and either the total serum IgE concentration or percent predicted FEV₁. Taking a genome-wide approach to DNA methylation and the serum concentration of IgE among asthmatic patients, we identified 11 IgE-associated DMRs (Fig 2, A, and see Table E8, A, in this article's Online Repository at www.jacionline.org), including a glutathione S-transferase (*GSTT1*). Focusing on the 81 asthma-associated DMRs (Fig 1, A), we found that 4 of these DMRs were associated with serum IgE concentrations ($P < .05$, Sidak corrected; Table II), and only one of those DMRs (*TRMT12*) overlapped with the 11 IgE-associated DMRs in the genome-wide analysis (Fig 2, A). We also investigated the relationship between DNA methylation and percent predicted FEV₁ among asthmatic patients and found that 16 DMRs were identified in the genome-wide DNA analysis (Fig 2, B, and see Table E8, B), and 28 DMRs were associated with FEV₁ when the analysis was limited to the 81 asthma-associated DMRs (Fig 1, A).

We next evaluated the relationship between asthma-, IgE-, or FEV₁-associated DMRs and gene expression. Gene expression analysis identified several interesting genes associated with asthma (T-box transcription factor, arachidonate 15-lipoxygenase, and NK cell genes) or the serum concentration of IgE among asthmatic patients (arachidonate 15-lipoxygenase) but not with percent predicted FEV₁ (see Fig E3 and Table E9 in this article's Online Repository at www.jacionline.org). Correlation of methylation and expression revealed enrichment in expected relationships with hypomethylated genes associated with increased gene expression and hypermethylated genes associated with decreased gene expression (binomial $P < .6 \times 10^{-11}$ for enrichment in inverse relationships in asthma-related DMRs [Fig 3, A] and $P < .01$ for IgE-related DMRs [Fig 3, B]); however, a number of DNA methylation changes were not associated with canonical gene expression differences (Fig 3, A and B). This was not the case for FEV₁ (see Fig E4 in this article's Online Repository at www.jacionline.org).

We also examined whether location of the DMR (upstream, gene body, or downstream of the gene) affected this relationship of methylation and expression. Most DMRs (85% [n = 69] for asthma and 82% [n = 9] for IgE) are in gene bodies, and only a few (4 for asthma and 1 for IgE) are upstream or downstream within 3 kb of the gene; therefore we were unable to detect any differences in methylation-expression relationships based on DMR location relative to gene. Of note, some DMRs are not associated with changes in expression (those close to zero on the y-axis in Fig 3, A and B, as well as a few intergenic DMRs that are >3 kb from any gene). However, among the DMRs within 3 kb of a gene, inverse correlations and gene expression are enriched in DMRs with higher statistical significance. We examined the percentage of inverse relationships in the 4 quartiles of DMRs ranked by adjusted (SLK-Sidak) P values and obtained the following results: 89% in the top quartile, 84% in the second quartile, 74% in the third quartile, and 55% in the bottom quartile. Additionally, the T_H2 immunity genes *IL13* and *RUNX3* are also among the genes with inverse correlation of DNA methylation and gene expression. This analysis demonstrates

that these inverse relationships rank at the top of asthma-associated DMRs and adds further biological relevance of our results. The finding that many of the inverse relationships are within gene bodies is in agreement with a recent publication that showed prominent hypomethylation of gene bodies with both inverse and direct correlation to gene expression in cancer⁵⁰ and demonstrates that the relationship of gene body methylation and expression is more complex than previously thought.⁵¹

To further explore the relationship between DNA methylation and gene expression, we performed an integrative analysis of DNA methylation and expression using LCMix⁴⁴ and identified 2484 methylation-expression pairs (posterior probability > 0.95 from the joint fit corresponding to a local false discovery rate < 0.05), including *RUNX3* and *IL4*, the expression of which was related to asthma-associated DNA methylation marks (Fig 3, C, and see Table E10 in this article's Online Repository at www.jacionline.org). Among the genes with the most significant methylation-expression relationship is an additional asthma-associated gene, *ST2*, the receptor for IL-33.^{52,53} Importantly, *ST2* is one of the few genes that has consistently been associated with asthma in genome-wide association studies.^{54,55}

We selected 10 loci from the 81 asthma-associated DMRs for internal and external validation by using pyrosequencing and an independent population of asthmatic children compared with our original control samples (Table I). Five loci were selected based on the relevance to asthma (*IL13*, *RUNX3*, *CAT*, *PRF1*, and *TIGIT*), 4 were selected based on large methylation differences between asthmatic patients and control subjects from the original comparison (*ALPL*, *C2/ZBTB12*, *KLF6*, and *NBR2*), and 1 locus (*IL4*) was selected based on the asthma-specific methylation-expression matrix. All 10 loci (13 CpG motifs) were validated internally in the original study population ($P < .05$, 1-tailed t test) comparing methylation changes detected by using the Illumina array and pyrosequencing assay (see Table E11 in this article's Online Repository at www.jacionline.org). As expected based on previous comparison of Illumina and pyrosequencing data,⁵⁶ there are some differences in percentage methylation change detected on the 2 platforms, but directionality and statistical significance are consistent. Five loci (*RUNX3*, *IL4*, *CAT*, *KLF6*, and *NBR2*, involving 8 CpG motifs) were validated externally in an independent population of children with asthma ($P < .05$, 1-tailed t test; see Table E11).

DISCUSSION

Our results demonstrate that methylation marks at specific loci are associated with asthma and define novel methylation-gene transcription relationships that might prove important in asthmatic patients. The relevance of epigenetic marks in asthmatic patients builds on our current understanding that the cause of asthma is associated with non-Mendelian inheritance, parent-of-origin patterns of inheritance, environmental exposures, and *in utero* exposures. Our findings that epigenetic marks in immune cells are associated with allergic asthma in inner-city children provide a provocative new direction for asthma research that is likely to have mechanistic, preventative, therapeutic, and public health implications and might establish novel biological insights into the development of asthma.

Our results suggest that epigenetic marks might prove important in understanding the pathogenesis of asthma. The asthma-associated DMRs appear to be biologically important; almost all were hypomethylated,⁵⁷ the majority were within gene bodies or CpG islands,⁵⁸ approximately 15% were in CpG island shores,⁵⁹ and asthma-associated DMRs were associated with gene expression. Moreover, we found that 3 genes previously associated with T_H2 immunity⁶⁰ and asthma pathogenesis,^{61,62} *IL4*, *IL13*, and *RUNX3*, are hypomethylated in asthmatic patients. Although IL-4 and IL-13 are classic T_H2 cytokines,^{60,61} *RUNX3* is a transcription factor known to regulate CD4/CD8 T-lymphocyte development by interacting with T-box transcription factor and silencing IL-4 expression.⁶³ Differential methylation of *RUNX3* has previously been associated with lung cancer,⁶⁴ as well as *in utero* exposure to cigarette smoke.⁶⁵ Moreover, the previously unrecognized asthma-associated epigenetic marks and methylation-expression relationships that we observed might identify novel genes and networks that are critically involved in the complex pathogenesis of this disease. Methylation changes in *KLF6* are of particular relevance given its role in activation of inducible nitric oxide synthase 1 during influenza A virus infection⁴⁸ and TGF- β during respiratory syncytial virus infection⁴⁹ and the importance of viral infections in childhood asthma.⁶⁶

Our results add to a growing body of evidence that methylation marks direct the expression of genes that are critical to the development of T_H2 immunity and allergic airway disease. *In vitro* development of mature T_H2 cells has been associated with hypomethylation of CpG motifs in the promoters of *IL4* and *IL13*^{11,12,67} and the RAD50-hypersensitive site in the T_H2 cytokine locus control region. Similarly, development of T_H1 immunity is also influenced by methylation. For example, cord blood CD4⁺ cells enhance development of the T_H1 lineage through progressive demethylation of the *IFNG* promoter.⁶⁸ DNA methylation of promoter and enhancer elements of forkhead box protein 3 has been shown to influence the development of regulatory T cells.¹³ *In vivo* experimentally induced methylation changes in CD11c⁺ dendritic cells,⁶⁹ CD19⁺ B lymphocytes,⁷⁰ *Ifng* in CD4⁺ cells,⁷¹ and *Runx3* in splenocytes and lung tissue¹⁰ have been observed in murine models of allergic airway disease. Demethylation with 5-aza-deoxycytidine demonstrates the importance of methylation marks in the development of T_H2 immunity¹⁰ and allergic airway disease.⁷¹

Our results could potentially affect our approach to asthma prevention and treatment. Although mutations in epigenetic regulators and epigenetic signatures have been observed in hematologic malignancies and are being targeted therapeutically,⁷² interest in using pharmacologic modulation of epigenetic marks in patients with nonmalignant diseases is only beginning to emerge.⁷³ Because epigenetic mechanisms are dynamic and reversible, it is entirely conceivable that a T_H2-skewed phenotype could be reprogrammed therapeutically by altering the methylome with small-molecule inhibitors that target specific methylation sites in the genome.⁷³ Moreover, the pharmacologic agents used to treat airway disease might function through epigenetic mechanisms; theophylline has been shown to affect histone deacetylase activity and improve steroid responsiveness.⁷⁴

There are several limitations to our study. The main limitation is the use of PBMCs as “surrogate” tissue for clinically and biologically relevant changes that occur in the lung. Although nasal epithelial cells might better reflect changes in airway epithelia, as has

recently been demonstrated for gene expression,⁷⁵ PBMCs are the most reasonable surrogate for examination of methylation changes related to immune phenotypes in asthmatic patients and are the most accessible and least invasive to collect in children. Moreover, atopic asthma can be considered a systemic immunologic derangement with a dramatic manifestation in the pulmonary airways, further supporting the significance of our findings in PBMCs. Methylation changes we identified in PBMCs are small in magnitude but might be reflective of larger changes in the lung. Alternatively, they might be small due to the presence of multiple cell types in PBMCs; however, further studies will be needed to demonstrate this.

Second, although we were unable to evaluate the effect of exposures on the epigenome because of lack of exposure assessment in our study, this is an important future direction for the field.

The third limitation is reliance on a statistical deconvolution method to assess differences in cell composition in the absence of white blood count data.

The fourth limitation is the inability to detect differences in methyl- versus hydroxyl-methyl cytosine, which is believed to be a mark for demethylation,⁷⁶ as well as changes in DNA methylation over time, because they are dynamic and influenced by exposures.

Finally, our association analysis is unable to distinguish between DNA methylation marks and gene expression being the cause or consequence of disease.

Despite these limitations, our findings that the epigenome is dysregulated in asthmatic patients provide a provocative new direction for this disease that might have important public health implications and might establish novel biological insights into the development of asthma. Although we identified strong associations between DNA methylation and gene expression, much more work will be needed to understand the complexities of this important regulatory mechanism in asthmatic patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

DMR	Differentially methylated region
NK	Natural killer
PC	Principal component
PCA	Principal components analysis

PEER Probabilistic estimation of expression residuals
RUNX3 Runt-related transcription factor 3

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Key messages

- DNA methylation changes in genes with direct relevance to T_H2 immunity and asthma are associated with allergic asthma in African American inner-city children.
- DNA methylation changes are associated with changes in gene expression with enrichment in inversely correlated methylation-expression relationships.
- Our results suggest that the epigenome is dysregulated in asthmatic patients, and this observation provides a provocative new direction for this disease that might have important mechanistic, therapeutic, and public health implications.

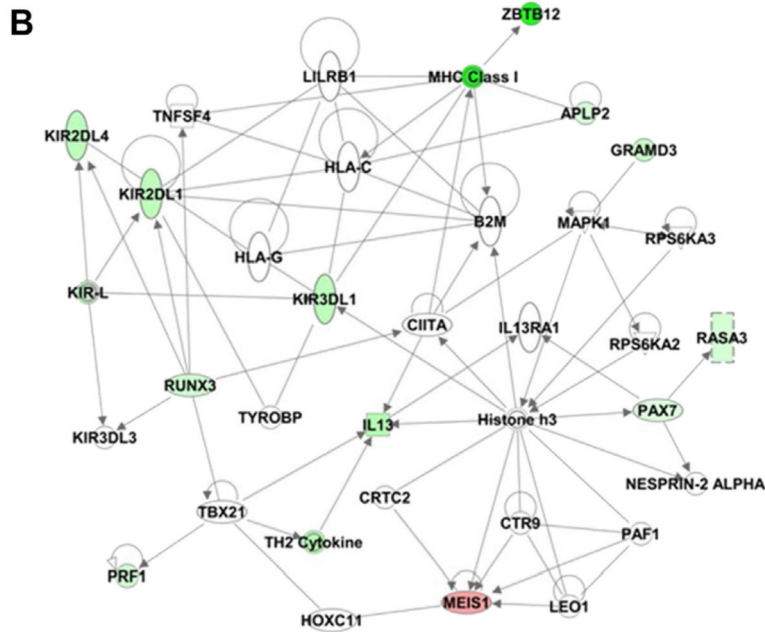
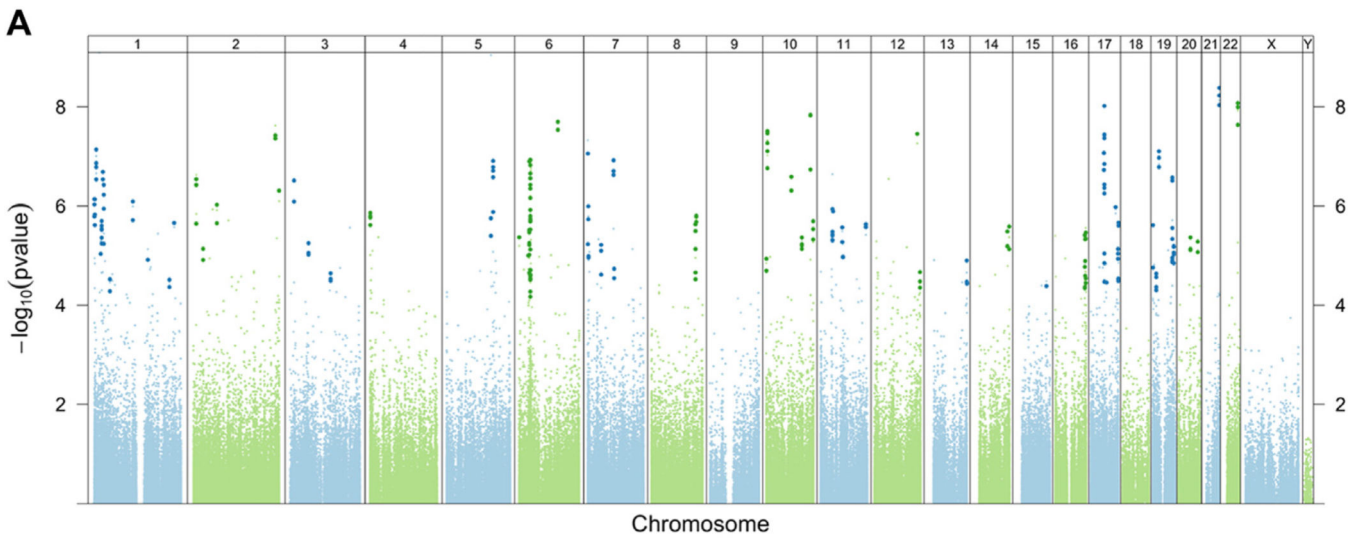


FIG 1. DMRs are associated with asthma after controlling for age, sex, race, technical variables, and batch effects. **A**, Manhattan plot of adjusted P values for disease status (asthma/control) from the linear model. Each *dot* represents a P value for a probe on the Illumina 450k array that has been adjusted by the significance of neighboring probes within 300 bases according to their correlation. Probes within statistically significant DMRs are identified by a darker and slightly larger symbol after adjustment for genome-wide comparisons. **B**, A representative transcriptional network of genes with associated DMRs from Ingenuity Pathway Analysis. Network analysis was performed with only direct interactions (*solid lines*) and networks with a score of greater than 20. Genes are intensity colored red (hypermethylated) or green (hypomethylated). *Horizontal ellipse*, Transcriptional regulator;

square, cytokine; *double circle*, group/complex; *triangle*, phosphatase; *vertical ellipse*, transmembrane receptor; *rectangle*, ion channel.

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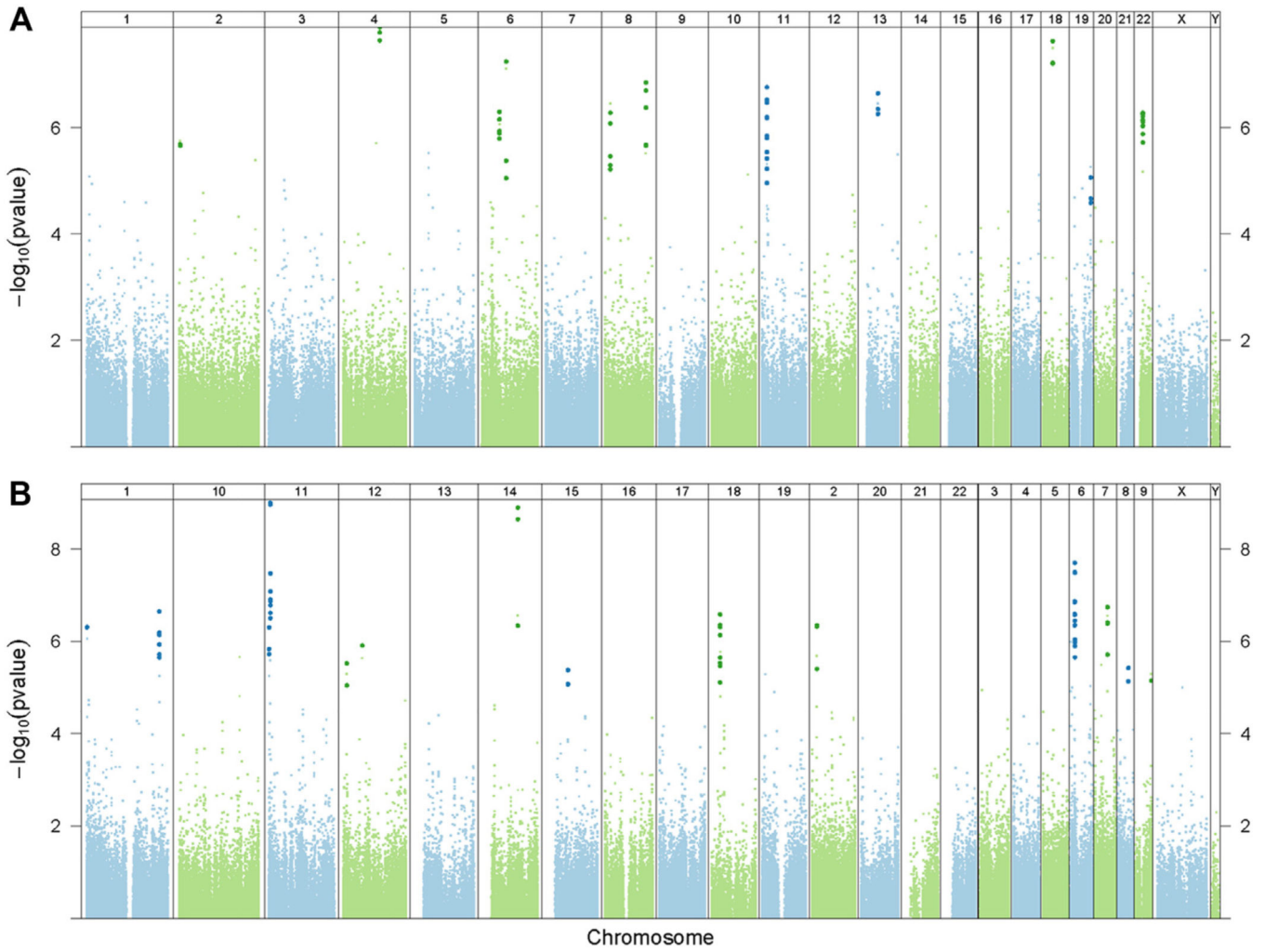


FIG 2. Differentially methylated marks are associated with higher total serum IgE concentrations (A) and percent predicted FEV₁ (B) after controlling for age, sex, race, technical variables, and batch effects among the asthmatic patients. Manhattan plots were constructed in the same fashion as in Fig 1, A Probes within statistically significant DMRs are identified by a darker and slightly larger symbol after adjustment for genome-wide comparisons.



FIG 3. Expression changes in genes within 3 kb of the nearest DMR associated with asthma in the entire study population (A), IgE levels among asthmatic patients (B), and genome-wide methylation expression in asthmatic patients (C). Fig 3, A, *x-axis*, The methylation difference is represented by the mean percentage methylation difference in asthmatic patients compared with control subjects. Fig 3, A, *y-axis*, The expression difference is represented by the mean fold change in asthmatic patients compared with control subjects (on the log base 2 scale). Fig 3, B, The percentage methylation difference on the *x-axis* is

represented by the mean correlation coefficient between IgE and methylation in asthmatic patients. The *y-axis* is represented by the slope of the IgE covariate in the linear model for expression in asthmatic patients. *Blue symbols* represent hypomethylated genes associated with increased gene expression, as well as some hypermethylated genes associated with decreased gene expression. *Red symbols* represent methylation changes that were not associated with expected gene expression differences. *Upward triangles* indicate DMR location upstream of the gene, *circles* represent DMRs in the gene body, and *downward triangles* refer to DMRs downstream of the gene. In Fig 3, C, the *t* statistic for expression change of all genes in the genome in asthmatic patients compared with control subjects from PBMCs is plotted against the *t* statistic for methylation changes in these genes (single CpG within the 3-kb promoter). *Red dots* represent the 207 genes with posterior probability from the joint model of methylation and expression of greater than 0.95 (corresponding to $q < 0.05$), a methylation *t* statistic of less than 21.5, and an expression *t* statistic of greater than 1.5 (see Table E10, A). Additionally, 82 genes have posterior probability of greater than 0.95, a methylation *t* statistic of less than 21.5, and an expression *t* statistic of less than 21.5 (*lower left quadrant*, see Table E10, B). No significant expression/methylation changes were identified in the *right 2 quadrants* on this plot.

TABLE I

Demographic and clinical features of asthmatic patients and control subjects

	Asthmatic patients (n 5 97)	Control subjects (n 5 97)	P value*	Validation asthmatic population (n 5 101)
Site				
Boston	23 (23.7%)	31 (32.0%)	<.01 [†]	13 (12.9%)
Dallas	8 (8.2%)	2 (2.1%)		10 (9.9%)
Denver	28 (28.9%)	13 (13.4%)		4 (4.0%)
Detroit	4 (4.1%)	6 (6.2%)		12 (11.9%)
New York	22 (22.7%)	14 (14.4%)		6 (5.9%)
Washington, DC	12 (12.4%)	31 (32.0%)		9 (8.9%)
Baltimore	–	–		28 (27.7%)
Chicago	–	–		10 (9.9%)
Cincinnati	–	–		9 (8.9%)
Age at recruitment (y)	9.0 (8.0–11.0)	9.0 (8.0–11.0)	.76 [‡]	9.0 (8.0–11.0)
Sex (male)	56 (57.7%)	41 (42.3%)	.03 [†]	64 (63.4%)
Participant race: African American (yes)	82 (84.5%)	86 (88.7%)	.40 [†]	101 (100.0%)
Participant race: Hispanic or Latino (yes)	26 (26.8%)	15 (15.5%)	.05 [†]	0 (0.0%)
How often exposed to smokers: daily	26 (26.8%)	25 (25.8%)	.85 [†]	22 (21.8%)
Dog living in the home in the last 6 mo (yes)	24 (24.7%)	22 (22.7%)	.74 [†]	24 (23.8%)
Cat living in the home in the last 6 mo (yes)	15 (15.5%)	24 (24.7%)	.11 [†]	12 (11.9%)
Any water/dampness in the last 12 mo (yes)	31 (32.0%)	16 (16.5%)	.01 [†]	30 (29.7%)
Gas stove, gas range, or gas oven (yes)	55 (56.7%)	69 (71.1%)	.04 [†]	61 (60.4%)
Allergic to any indoor allergen (yes)	97 (100.0%)	0 (0.0%)	§	96 (95.1%)
Cockroach IgE (IU/mL)	0.34 (0.34–1.65)	0.34 (0.34–0.34)	§	0.62 (0.34–10.0)
Total IgE (kU/L)	408.5 (189.0–951.0)	29.0 (15.0–58.0)	§	481.0 (167.0–1264.0)
Baseline: FEV ₁ (% predicted)	90.9 ± 16.7	103.0 ± 10.3	§	93.9 ± 19.3
Baseline: FEV ₁ /FVC ratio	75.5 ± 9.7	84.4 ± 6.1	§	77.5 ± 10.2
Asthma medications				
Albuterol	95 (97.9%)	0 (0.0%)	§	101 (100.0%)
Inhaled steroids	84 (86.6%)	0 (0.0%)		84 (83.2%)
Montelukast	38 (39.2%)	0 (0.0%)		9 (8.9%)

Data are presented as means ± SDs, medians (IQRs), or numbers (percentages).

FVC, Forced vital capacity.

* P values were calculated with the use of the χ^2 test for categorical variables and the Wilcoxon rank sum test for continuous variables.

[†] χ^2 Test.

[‡] Rank sum test.

§By protocol design.

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TABLE II

DMRs associated with asthma at an SLK-Sidak *P* value of less than .05

Chromosome	Start	End	No. of probes	Asthma SLK <i>P</i> value	Asthma SLK-Sidak <i>P</i> value	Gene	Location	CpG island distance	Methylation difference (asthma-control [%])	FEV ₁ <i>P</i> value	IgE <i>P</i> value
chr1	1091164	1091325	4	5.59E-07	.00168	CS444397	1075	717	-0.012	.003504	
chr1	2220460	2220783	4	4.52E-07	.00068	SKI	Intron	0	-0.015		
chr1	6263726	6263914	4	3.79E-08	9.79E-05	ACOT7	Exon;intron;nc_exon;nc_intron	0	-0.011		
chr1	6277594	6277647	2	1.41E-07	.0013	ACOT7	Exon;intron;nc_exon;nc_intron	0	-0.02	.03596	
chr1	15264894	15265020	2	4.38E-10	1.69E-06	LOC391005	Intron;utr3	0	-0.029	.03778	
chr1	18883602	18883759	2	3.83E-06	.01177	PAX7	Intron	0	-0.017		
chr1	21750110	21750368	5	3.59E-06	.00673	ALPL	utr5_intron	23,263	-0.01		
chr1	21773573	21773847	3	3.79E-06	.00668	ALPL	Intron	0	-0.03		
chr1	25113451	25113525	3	1.63E-07	.00107	RUNX3	Intron	0	-0.018		
chr1	27555725	27556088	5	1.96E-07	.00026	MAP3K6	Exon;intron	0	-0.018		
chr1	45046081	45046267	3	1.74E-05	.04438	TCTEX1D4	-537	1,268	0.006	.01963	
chr1	110108029	110108165	3	5.01E-07	.00179	EPSSL3	utr5	23,218	-0.01	.01368	
chr1	152566740	152566865	2	5.46E-06	.021	ATP8B2	utr5_intron	807	0.012	.03256	
chr1	213807046	213807324	3	2.66E-05	.0453	KCTD3	34	0	0.002		
chr1	227230174	227230200	2	2.00E-06	.03665	AX748369	-110597	119,496	0.01		
chr2	8339686	8339783	3	9.66E-08	.00048	LINC00299	nc_intron	77,665	-0.019	.03115	
chr2	8340572	8340606	2	1.02E-06	.01438	LINC00299	nc_intron	76,842	-0.017		
chr2	27652710	27652915	3	7.47E-06	.01754	C2orf16	utr5	6,296	-0.013		
chr2	66518038	66518302	3	4.82E-07	.00089	MEIS1	Intron	1	0.009		
chr2	232101307	232101500	3	1.15E-08	2.90E-05	NMUR1	Exon	0	-0.018	.0002612	
chr2	242516227	242516284	2	5.56E-07	.00472	AK097934	45223	707	-0.025		
chr3	11626535	11626881	3	1.25E-07	.00018	VGLL4	Intron	0	-0.012		
chr3	52787406	52787560	4	5.39E-06	.01686	ITIH1	Exon;nc_exon	7,238	-0.013		

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chr3	115495348	115495602	4	1.67E-05	.03144	<i>TIGIT</i>	Exon;utr5	16,590	-0.01		
chr4	1233848	1234086	7	5.29E-07	.00108	<i>AK125775</i>	nc_exon	0	-0.018		
chr5	125723585	125723747	3	3.32E-10	9.96E-07	<i>GRAMD3</i>	63253	63,229	-0.012	.03647	
chr5	132021512	132021960	6	5.34E-08	5.79E-05	<i>IL13</i>	Exon;intron;utr5	1,309	-0.008		
chr6	458210	458297	2	4.91E-06	.027	<i>EXOC2</i>	Intron	2,395	-0.014		
chr6	26334181	26334235	2	1.41E-07	.00126	<i>HIST1H3F</i>	utr3	0	-0.009		
chr6	29019737	29020145	10	8.39E-07	.001	<i>TRIM27</i>	-19990	0	0.017		
chr6	31954747	31955007	10	6.90E-06	.01281	<i>SLC44A4</i>	utr5	0	-0.015		
chr6	31975676	31976004	20	2.89E-08	4.28E-05	<i>C2:ZBTB12</i>	Exon;utr3;utr5_intron	0	-0.015	.01655	
chr6	109881430	109881854	3	1.42E-08	1.62E-05	<i>MICAL1</i>	Exon;intron	942	-0.008		
chr7	888576	888761	3	3.47E-08	9.11E-05	<i>GET4;SUN1</i>	Intron;nc_intron	0	-0.005		
chr7	2056125	2056406	3	7.89E-07	.00136	<i>MADI1; MAD1L1</i>	Intron	0	-0.016	.01102	
chr7	2695301	2695438	3	8.55E-06	.02983	<i>AMZ1</i>	utr5_intron	0	-0.013		
chr7	38323253	38323572	4	2.71E-06	.00412	<i>TCRg:TRGV9</i>	Exon;intron;nc_exon	5,468	-0.021		
chr7	73283658	73283902	4	9.19E-08	.00018	<i>RFC2</i>	utr3	0	-0.017		
chr7	74890962	74891132	3	1.05E-05	.0296	<i>POM121C</i>	Intron	0	-0.01	.00866	
chr8	125532162	125532491	6	1.73E-06	.00255	<i>TRMT12</i>	Exon;utr5	0	0.016	9.29E-08	
chr8	127638762	127638859	4	8.24E-07	.00412	<i>FAM84B</i>	Exon;utr5	0	-0.008	.0131	
chr10	848816	848923	3	8.24E-06	.0367	<i>LARP5</i>	Exon;utr3	1,721	-0.012	.03864	
chr10	3813757	3814016	5	8.39E-09	1.57E-05	<i>KLF6</i>	Exon;intron	0	-0.021		
chr10	3814386	3814687	4	3.08E-08	4.96E-05	<i>KLF6</i>	Exon;intron	88	-0.024		
chr10	72030353	72030454	3	1.27E-07	.00061	<i>PRF1</i>	Exon	0	-0.015	.01763	
chr10	101814909	101815175	5	3.92E-06	.00713	<i>CPN1</i>	Exon;intron	0	-0.016		
chr10	126320834	126321170	4	2.03E-07	.00029	<i>FAM53B</i>	Intron	246	-0.016	.0125	
chr10	134072207	134072497	4	2.63E-06	.0044	<i>PWWP2B</i>	Intron;utr3_intron	0	-0.009	.00745	

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chr11	34416873	34417133	7	7.88E-07	.00147	CAT	utr5	0	-0.018	.01748	
chr11	36377805	36377877	2	7.07E-07	.00475	FLJI4213	Intron:utr5_intron	21,831	-0.01	.006936	
chr11	62378748	62378810	3	1.62E-06	.01262	SNHG1	nc_intron	696	0.007		
chr11	64289922	64290199	3	3.71E-06	.00647	SF1	Exon:intron	0	-0.009	.02808	
chr11	129485687	129485739	3	2.10E-06	.01937	APLP2	Exon:utr5_intron	13,029	-0.007	.0003045	
chr12	122162107	122162366	2	3.43E-08	6.43E-05	PITPNM2	utr5_intron	38,347	-0.021		
chr12	130134393	130134600	4	1.33E-05	.03062	GPR133	Intron:utr5_intron	0	-0.006	.0001811	
chr13	113220890	113221025	3	1.19E-05	.04192	TMCO3	Intron	0	-0.015		
chr13	113825056	113825282	3	1.34E-05	.02828	RASA3	Intron:utr5_intron	0	-0.007		
chr14	98857115	98857373	3	3.11E-06	.00583	BCL11B	-49540	0	-0.015		
chr14	104255101	104255221	3	2.80E-06	.01125	INF2	Intron:nc_intron; utr3_intron	0	-0.013		
chr15	89170960	89171237	2	2.72E-05	.0465	BLM	-11270	0	-0.013	.0374	
chr16	84108978	84109249	3	1.59E-06	.00285	KIAA0182	93281	0	-0.01		
chr16	84233792	84234027	4	1.86E-05	.03767	KIAA0182	Intron:utr5_intron	0	-0.011		
chr16	85272036	85272335	4	1.63E-06	.00264	BC041439	39457	0	-0.012		
chr16	87075079	87075362	3	1.39E-05	.02358	ZFPPI1	Intron	1,919	-0.003		
chr16	87085565	87085738	3	1.59E-06	.00444	ZFPPI1	Intron	0	-0.018		
chr17	37379133	37379307	3	5.9E-08	.00016	CNP	Exon	0	-0.015	.00594	
chr17	38531660	38531955	12	1.59E-08	2.62E-05	NBR2	nc_intron	0	-0.021	.02746	
chr17	44652266	44652511	2	1.18E-05	.02301	ABI3	Exon:intron	0	-0.014	.002791	
chr17	70268887	70268897	2	9.75E-07	.04621	SLC9A3R1	Intron:nc_intron	11,350	-0.017	.005602	
chr17	77226056	77226266	4	4.00E-06	.00921	TSPAN10	nc_exon:utr3	0	-0.031		
chr17	77986792	77986955	4	1.35E-05	.03938	HEXDC	Exon:intron	0	-0.011	.0008078	
chr17	78474047	78474241	3	1.15E-06	.00288	TBCD	Intron	0	-0.013		
chr19	1207998	1208217	3	1.38E-05	.03013	MIDN	Exon	0	-0.007		
chr19	10523581	10523782	5	1.46E-05	.03466	ATG4D	Exon:intron:nc_exon:nc_intron; utr5	3,012	-0.008		

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chr19	18121329	18121515	4	3.31E-08	8.64E-05	MAST3	Exon	0	-0.016		
chr19	55911909	55912349	9	1.02E-07	.00011	SHANK1	Exon;utr5	0	-0.008	7.29E-06	
chr19	60006690	60006895	6	3.44E-06	.00812	KIR2DL4; KIR2DS2; KIR3DL1	Intron;utr5	12,754	-0.012		
chr20	34937784	34937967	4	1.33E-06	.00353	C20orf118	17	11,603	-0.008		
chr20	55416108	55416384	3	3.23E-06	.00566	RBM38	Exon;utr3	0	-0.012	.0143	
chr21	46670037	46670291	4	1.79E-09	3.42E-06	PCNT	Exon;intron	0	-0.021	.00112	
chr22	45064135	45064392	4	3.11E-09	5.88E-06	TTC38	Exon;intron	0	-0.023		