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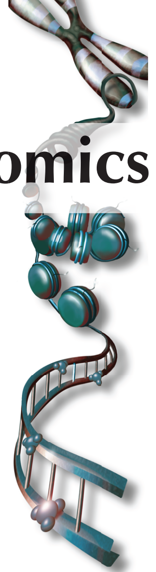

Nasal DNA methylation is associated with childhood asthma

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Aim: We aim to study DNA methylation (DNAm) variations associated with childhood asthma. **Methods:** Nasal DNAm was compared between sibling pairs discordant for asthma, 29 sib pairs for genome-wide association studies and 54 sib pairs for verification by pyrosequencing. Associations of methylation with asthma symptoms, allergy and environmental exposures were evaluated. *In vitro* experiments and functional genomic analyses were performed to explore biologic relevance. **Results:** Three CpGs were associated with asthma. cg14830002 was associated with allergies in nonasthmatics. cg23602092 was associated with asthma symptoms. cg14830002 and cg23602092 were associated with traffic-related air pollution exposure. Nearby genes were transcriptionally regulated by diesel exhaust, house dust mite and 5-aza-2'-deoxycytidine. Active chromatin marks and transcription factor binding were found around these sites. **Conclusion:** We identified novel DNAm variations associated with childhood asthma and suggested new disease-contributing epigenetic mechanisms.

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Keywords: air pollution • childhood asthma • DNA methylation • exposures • functional genomics analysis • histone marks • sibling study • transcriptional factor

From 2001 to 2013, the prevalence of asthma in children aged 0–17 years was 8–10% on average [1]. Throughout this time period, the adjusted prevalence among poor children was higher than near-poor and non-poor children. Significant racial disparities still remain, and African–American (AA) children have much higher asthma prevalence than white children [1]. Common genetic variants only explain a small portion (∼5%) of asthma heritability [2]. Environmental exposures also play a role; traffic-related air pollution (TRAP) and secondhand smoke (SHS) have been found to increase asthma prevalence and severity [3–5]. For example, *in utero* exposure to tobacco smoke is associated with childhood asthma, and this exposure can modify gene expression through DNA methylation (DNAm) [6,7].

During recent years, epigenetic marks have emerged as a potential mechanism explaining the non-Mendelian [8] and parent of origin patterns of asthma [9,10]. In addition, epigenetic marks may also explain the short-term and long-term (even transgenerational) effects of environmental exposures in asthma [11–13]. Epigenetic marks regulate many processes of immune cells involved in asthma, particularly T lymphocytes (T_H1, T_H2 and regulatory T cells) [13]. Several small-scale epigenome-wide association studies have successfully identified DNAm of particular nucleotides as a biomarker for asthma, suggesting candidate pathways involved in asthma [14–20]. Four of these studies were conducted in nasal epithelial cells, a proxy for the lower airway epithelium in gene expression and DNAm studies [17–20]. The airway epithelium plays a key role in asthma development due to its unique interface with

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the environment and interaction with immune cells [21–23]. DNAm markers for particular asthma characteristics and subgroups, such as serum IgE levels [24], eosinophilic/paucigranulocytic/neutrophilic asthma [25], asthma that develops in childhood and persists into early adulthood [26] and markers for temporal asthma transition [27] have also been identified in blood or saliva.

In the present study, we aimed to identify DNAm markers in the nasal epithelium that are differentially methylated between AA siblings who were 5–18 years old and discordant for asthma. The usage of siblings allows us to better control confounding environmental factors that may modify DNAm and contribute to asthma, including TRAP and SHS, which may increase the likelihood of identifying markers with small effects that are related to childhood asthma. Additionally, we examined the impact of exposure such as TRAP and SHS on asthma-associated DNAm variation and studied whether these markers are also associated with allergy and asthma symptoms.

Methods

The online Supplementary Materials provide additional details on methods.

Study population

The Exposure Sibling Study (ESS) was approved by the Institutional Review Board at Cincinnati Children's Hospital Medical Center (CCHMC). Written informed consent was obtained from each participant or the participant's parent or legal guardian before study participation. ESS is a cross-sectional study consisting of AA siblings between age 5 and 18 years. Eligible siblings were nontwins, discordant for asthma and born and raised in the same household in the Cincinnati Metropolitan area. Asthma diagnosis was obtained from the parental report, and confirmed via the electronic medical record that the diagnosis is current. Nasal samples were collected at the same visit for the siblings as described below and height and weight were recorded. All nasal samples contained >90% epithelial cells similar to our previous findings [28]. Electronic survey (Research electronic data capture [REDCap] software) [29] captured information including asthma onset, diagnosis, symptoms, severity, quality of life, medication, environmental exposures, social histories and residential address for the first year of life and for the past 5 years. To be cost-effective and more focused on promising candidates, only the initial 29 sibling pairs with sufficient DNA were included for genome-wide analysis. Sample size analysis using the array data showed that 51 sib pairs had 80% power to detect the DNAm difference between control and asthmatics for the selected CpG sites. Therefore, the following available 25 sibling pairs were added for pyrosequencing (54 sib pairs in total). All samples are full siblings. Elemental carbon attributable to traffic (ECAT; approximately 95% of which is diesel exhaust particles (DEP) [30], herein referred to as TRAP) was estimated using previously established methods [31]. Two estimates were generated: birth or early exposure to TRAP was defined as exposure during the first year of life (birth ECAT); and current exposure was derived using the current address (current ECAT). Children with ≥95th BMI percentiles (considering age and sex based on the Centers for Disease Control and Prevention curves) were considered obese [32].

The Genomics of Secondhand smoke Exposure in Pediatric asthma study (GSEP) was also approved by the Institutional Review Board at CCHMC. Written informed consent was obtained from each participant or the participant's parent or legal guardian before study participation. Asthmatic AA children aged 5–18 were screened from the Allergy and Pulmonary Clinics at CCHMC. SHS exposure was determined by questionnaire and confirmed by urine cotinine using TobacAlert™ and nasal samples were collected. In addition to GSEP participants, we also collected nasal samples from five unrelated, nonasthmatic subjects from an underserved community; all were exposed to SHS based on a coordinator-administered survey.

Nasal cell DNA processing and DNAm measurement by arrays and bisulfite pyrosequencing are described in the Online Data Supplement. Array data are deposited to GEO (GSE109446). Primers used for pyrosequencing and RT-qPCR are listed in Supplementary Tables 1 & 2.

Definition of allergy & asthma symptoms

Among the asthmatics, asthma severity was defined by symptom frequency using our previously validated respiratory symptom score [33]. Via questionnaire, the parent(s) of the participants reported how many times per week over the past 12 months the participant had cough without a cold, wheezing/high pitched sound in the chest, shortness of breath or chest tightness/pain. The frequencies were reported (and scored) as: never (0); less than 1 (1), 1–2 (2), 3–5 (3) and 6–7 (4)-times per week. The maximum respiratory symptom score (maxRSS) was calculated by taking the maximum score across the four symptom questions.

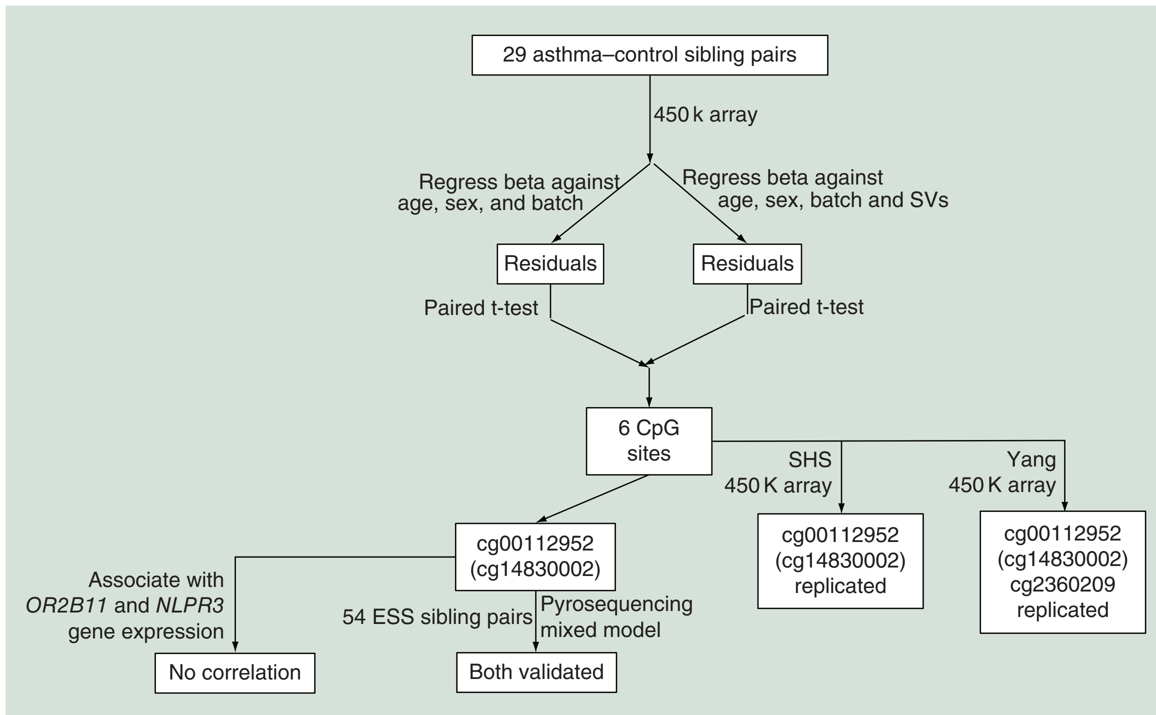

Figure 1. Analysis flow chart.
ESS: Exposure Sibling Study; SHS: Secondhand smoke; SV: Surrogate variables.

Statistical analysis

The analyses were performed as outlined in Figure 1. Before analyses, data quality and distributions were examined. To compare the demographics and characteristics between the asthmatic and nonasthmatic subjects, we used T, Wilcoxon rank sum and Fisher's exact tests according to the data distribution. To identify CpG sites that had differential methylation levels between asthmatics and their nonasthmatic siblings, we analyzed the beta values in the first set of 29 pairs of siblings. β values of each of the CpG sites were regressed against age, sex and batch as two batches were observed using multidimensional scaling. Residuals were generated and then compared between asthmatics and nonasthmatics using paired t-tests. To further correct for batch effects and bias caused by unknown confounders such as cell composition, we performed surrogate variable analysis using the R package 'surrogate variable analysis'. Three significant surrogate variables (SVs) were detected. Methylation levels of the asthmatic and nonasthmatic siblings were compared again as described above, but with three SVs being included in the regressions to get the residuals. Only CpG sites identified in both sets of analyses ($p < 0.05$) were selected for further examination. Genome-wide false discovery rate was estimated using R package 'q-value'. Association between DNAm and asthma was validated for candidate sites using pyrosequencing. As control and asthmatic siblings were from the same household, to account for the random family effect, a mixed model was used, in which age and sex were controlled for. The effects of current and birth ECAT, and allergy and asthma severity were also tested using the mixed models. Comparisons of expression between controls and 5-aza or DEP/house dust mite (HDM) treated groups in human bronchial epithelial cells (HBECs) were compared by two-sided t-test in GraphPad Prism (CA, USA).

Functional genomics studies

We collected genome-wide functional genomic datasets from a variety of sources, including ENCODE [34], Cistrome [35], PAZAR [36], Re-Map [37] and NIH Roadmap Epigenomics [38]. All datasets were indexed by their genomic coordinates, which were used to intersect with the genomic coordinates of methylation sites of interest.

Table 1. Population characteristic of Exposure Sibling Study cohort.

Characteristic	Asthmatics (n = 54)	Nonasthmatics (n = 54)	p-value
Age	12.01 (2.94)	11.35 (3.69)	0.31
Sex:			0.05
– Male	34 (63%)	23 (43%)	
Race:			1.00
– Black	52 (96%)	52 (96%)	
– White	0 (0%)	0 (0%)	
– Biracial	2 (4%)	2 (4%)	
BMI z score	0.81 (1.27)	0.58 (1.38)	0.37
SHS:			1.00
– Yes	28 (52%)	28 (52%)	
Current ECAT	0.35 (0.29–0.43)	0.35 (0.29–0.43)	1.00
Birth ECAT	0.42 (0.33–0.53)	0.45 (0.34–0.56)	0.57
Allergy:			<0.001
– Yes	42 (78%)	22 (41%)	
maxRSS	2 (1–3)	–	–

Age and BMI z score were shown as mean (SD) and compared using t-test; current ECAT, birth ECAT and maxRSS were shown as median (interquartile range) and compared using Wilcoxon rank sum tests; sex, race, SHS and allergy status were shown as frequencies (proportion) and compared using Fisher's exact tests. BMI z scores were calculated using CDC growth chart.
BMI: Body mass index; ECAT: Elemental carbon attributed to traffic; maxRSS: Maximum respiratory symptom score; SD: Standard deviation; SHS: Secondhand smoke.

Results

Population characteristics

The demographics and characteristics of the initial subjects consented to the ESS are shown in Table 1. All children were AA. No statistically significant differences were detected between asthmatics and their nonasthmatic siblings in age, sex, BMI z score or birth ECAT. As the sibling pairs were recruited from the same household, their race, SHS and current ECAT did not differ. Among all the subjects, a subset of 29 pairs was assayed by microarrays for discovery purposes. No significant differences were observed between the discovery set and the rest of the subjects (data not shown) except for age; the discovery set was 1 year older ($p = 0.038$, t-test). As expected, there were significantly more children with allergic symptoms among asthmatics ($p < 0.001$, Fisher's exact test).

DNAm variation in the nasal epithelium is associated with asthma

To identify asthma-associated DNAm, we performed Infinium 450 K Beadchip analysis on the nasal DNAs from 29 pairs of siblings discordant for asthma, as outlined in Figure 1. Genome-wide DNAm analysis identified six autosomal CpG sites whose methylation was nominally associated with asthma status ($p < 0.05$, mean paired difference in $\beta \geq 10\%$) (Table 2). None of these associations, however, reached genome-wide significance (q-values range 0.61–0.71).

All of the six sites are non-SNP CpGs (no co-localization of SNP from dbSNP142 with minor allele frequency (MAF) $\geq$1% at C or G), and four of them are located in promoters (within 1500bp of a gene transcription start site). For example, a CpG site located with the *OR2B11* promoter (cg00112952) exhibited substantial differential methylation between asthmatics and nonasthmatics. *OR2B11* encodes an olfactory receptor responsible for the recognition and G protein-mediated transduction of odorant signals, and its role in asthma and response to exposure is unknown. Interestingly, cg14830002, a CpG site located 163 bp downstream of cg00112952, also showed statistical significance, even though the difference in β was slightly lower than our cutoff ($\beta_{(asthma-control)}$ =-0.08). cg14007090 is located within an intron of *LAMA5*, which has been implicated in asthma development and lung function [39–41]. cg20223677 is located within the promoter of *DEF104A/DEFB104B*, which encodes β-defensin 104A/104B. In addition to their antimicrobial activity, β-defensins have multifaceted functions in innate and adaptive immunity [42]. They are expressed in most epithelial cells and have been shown to have impaired function in pulmonary inflammation [43,44]. cg26017880 is located within a CpG island in the *ATP9B* promoter. *ATP9B* encodes a putative aminophospholipid translocase and may be involved in ion transportation [45]. cg23602092, located in the promoter of *TET1*, has been shown to be associated with asthma in our previous study [17]. Since all these genes have relevant functions in asthma, we set out to replicate them in two different cohorts.

Table 2. Association of nasal DNA methylation with childhood asthma in Exposure Sibling Study, Genomics of Secondhand smoke Exposure in Pediatric asthma study and Inner City Asthma Consortium study.

CpG site	ESS 450 K (n = 58)			ESS pyro (n = 108)		GSEP EPIC (n = 41)		ICAC 450 K (n = 72)		Gene	Genomic coordinates	Relation to UCSC CpG island	Relation to TSS
	Difference[†]	p-value (no SV)	p-value (with SV)	Difference[‡]	p-value	Difference[†]	p-value	Difference[†]	p-value				
cg06193597	0.20	0.035	0.029			0.21	0.115	-0.12	0.148		Chr2: 241896910		
cg00112952	**-0.13**	**0.004**	**0.004**	**-8.19%**	**0.039**	**-0.21**	**0.006**	**-0.10**	**0.005**	*OR2B11*	Chr1: 247616523	S_Shore	TSS1500
cg14007090	-0.11	0.023	0.001			-0.10	0.327	-0.05	0.362	*LAMA5*	Chr20: 60915009	S_Shelf	Body
cg20223677	-0.11	0.050	0.036					-0.04	0.477	*DEFB104BDEFB104A*	Chr8: 7332846		TSS1500
cg26017880	0.11	0.018	0.017					0.01	0.396	*ATP9B*	Chr: 1876829239	Island	TSS200
cg23602092	**-0.11**	**0.016**	**0.011**	**-5.24%**	**0.028**	0.02	0.747	**-0.07**	**0.034**	*TET1*	Chr10: 70319645	N_Shore	TSS1500
cg14830002	**-0.08**	**0.001**	**0.002**	**-2.78%**	**0.020**	**-0.08**	**0.021**	**-0.04**	**0.092**	*OR2B11*	Chr1: 247616686	S_Shore	TSS1500

Verified and replicated CpG sites are highlighted in bold.

[†] Mean paired difference (asthma–control).

[‡] Least square means of the difference between asthma and control with the values of other co-variables set at mean levels of the cohort; p-values were from tests on the association of asthma with β-values or methylation%; analysis on the M values support the conclusion. Two CpG sites from the GSEP data did not pass QC thus being excluded form analysis. Hg19 was used for the gene annotations.

EPIC: Illumina Methylation EPIC arrays; ESS: Exposure Sibling Study; GSEP: Genomics of Secondhand smoke Exposure in Pediatric Asthma Study; ICAC: Inner City Asthma Consortium; QC: Quality control; SHS: Secondhand smoke; SV: Surrogate variable; TSS: Transcription start site; UCSC: University of California, Santa Cruz Genome Browser.

Table 3. Association of cg00112952, cg23602092 and cg14830002 with environmental exposures, allergy and asthma symptoms.

Characteristic	cg14830002			cg00112952			cg23602092		
	Overall p-value	Controls p-value	Asthmatics p-value	Overall p-value	Controls p-value	Asthmatics p-value	Overall p-value	Controls p-value	Asthmatics p-value
Current ECAT	–	0.005	ns	ns	ns	ns	–	<0.001	ns
Birth ECAT	ns	ns	ns	ns	ns	ns	0.040	ns	ns
SHS	ns	ns	ns	ns	ns	ns	ns	ns	ns
Allergy	–	0.025	ns	ns	ns	ns	ns	ns	ns
Asthma symptoms†	–	–	ns	–	–	ns	–	–	0.009

†Asthma symptom was tested in asthmatics only (see Methods).
When different effects were found in controls and asthmatics, respectively, the overall effects were not presented (labeled as –).
ECAT: Elemental carbon attributable to traffic; ns: Not significant; SHS: Secondhand smoke.

Replication & verification of ESS results

To confirm the association of asthma for these six CpG sites, we examined data from the GSEP study and the published Inner City Asthma Consortium (ICAC) study [18], both of which had case–control designs consisting of unrelated subjects. Supplementary Table 3 shows the demographics for the GSEP study, and the ICAC was previously described [18]. In the GSEP study, asthma was significantly associated with lower methylation levels of both cg14830002 ($p = 0.021$, $\beta_{(asthma–control)} = -0.08$) and cg00112952 ($p = 0.006$, $\beta_{(asthma–control)} = -0.21$) (Table 2). Since the five nonasthmatics were all SHS-exposed females, we performed analyses either stratified by sex or SHS exposure, or including sex and SHS as co-variables in the modeling to rule out confounding effects. The results showed that methylation of these two CpG sites were only associated with asthma status, but not sex or SHS exposure. Indeed, when the analysis was restricted only to SHS exposed females and the sample size was reduced to 17, significant association between asthma and cg00112952 was still detected ($p = 0.013$, $\beta_{(asthma–control)} = -0.16$). In contrast, the association of asthma with cg06193597, cg14007090 and cg23602092 was not replicated in GSEP. All the associations were also tested using M-values, with the conclusions remaining the same.

We further tested the associations detected by the ESS study using data from ICAC samples (Table 2) [18]. Upon controlling for random study site effects, the associations were significant for cg00112952 ($p = 0.005$, $\beta_{(asthma–control)} = -0.10$), and marginally significant for cg14830002 ($p = 0.092$, $\beta_{(asthma–control)} = -0.04$). Interestingly, the association between asthma and cg23602092 we previously published [17] was also replicated ($p = 0.0342$, $\beta_{(asthma–control)} = -0.07$). These conclusions were again supported by models based upon M-values. In total, the associations of asthma with DNAm levels at cg00112952 and cg14830002 were detected in three different cohorts.

Using the extended ESS cohort of 54 sibling pairs, we verified cg00112952, cg14830002 and cg23602092 by pyrosequencing. After adjusting for age, sex and other significant predictors, and accounting for random inter-family effects, asthmatics showed lower DNAm level in all three sites (Table 2).

Relationship between asthma-associated DNAm variation with exposures, allergy & asthma symptoms

In addition to asthma, we examined the association of DNAm at cg14830002, cg00112952 and cg23602092 with TRAP (measured by current ECAT [31]), birth ECAT and SHS (Table 3). Interaction terms were included to test asthma status-specific ECAT effects on DNAm. The methylation level of cg14830002 significantly increased with higher current TRAP exposure in controls (3% increase in methylation % with 0.1 unit increase in current ECAT), but not in asthmatics. No association with birth ECAT was detected. In cg23602092, consistent with our previous publication, the methylation level significantly increased when current TRAP exposure increases, but only in controls (9% increase in methylation % with 0.1 unit increase in current ECAT). In cg23602092, a marginal negative correlation with birth ECAT was detected. In contrast, no statistically significant association of methylation in cg00112952 was detected with either TRAP exposures. No association with SHS was found for any of the sites.

DNAm at cg00112952 and cg23602092 was not associated with allergic status (Table 3). cg14830002 was associated with allergy only in nonasthmatic controls ($p = 0.025$, $\text{difference}_{(allergic-nonallergic)} = -3.85\%$). As allergy and asthma status are highly correlated in our cohort (Table 1), to verify that the association between asthma and

cg14830002 is not due to this correlation, we tested the association between asthma and cg14830002 with allergy status stratified. A significant association was found in nonallergic (p = 0.023, $\text{difference}_{(\text{asthma–control})}$ = -4.77%) subjects, but not in allergic subjects. These results suggest that cg14830002 may play a role in both asthma and allergy.

Among the asthmatics, we also tested the association of asthma symptom frequencies (maxRSS, definition in Methods) with DNAm variation (Table 3). Though the methylation % of cg23602092 was comparable when maxRSS was 3 and below (14.2, 14.7, 13.1 and 17.7% when maxRSS is 0, 1, 2 and 3, respectively), it became significantly higher when maxRSS reached 4 (36.4%, p = 0.009).

Since cg14830002 was associated with current ECAT and allergy in controls, we acutely (0–24 h) exposed HBEC cells to various doses of HDM or DEPs, the major particulate matter in TRAP). Strikingly, two genes located close to cg14830002, *OR2B11* and *NLRP3*, both showed time- and dose-dependent expression changes to DEP exposure, but not to HDM (Figure 2A & B & Supplementary Figure 1A &1B). Specifically, at a relatively low dose of DEP the expression levels of *OR2B11* and *NLRP3* were significantly increased at the 24 h time point, while at a higher dose their expression was reduced at the same time point. This suggest that in response to DEP, the expression of *OR2B11* and *NLRP3* increases first and then decreases at a later time point; a higher dose of DEP causes this to occur earlier. However, no significant DNAm changes in cg00112952 or cg14830002 were observed in the exposed cells compared with controls (Supplementary Figure 2). In support of our previous finding [17], exposure to DEP and HDM significantly increased the expression of *TET1* at a very early time point (Figure 2C & Supplementary Figure 1C), which correlated with changes in cg23602092 methylation (Supplementary Figure 2).

Functional genomics analysis

We next sought to identify the functional impact of the DNAm variation we have identified. First, we examined the effect of the asthma-associated CpG sites on gene expression. Previously we demonstrated the regulation of *TET1* expression by cg23602092 [17]. We assayed the mRNA levels of *OR2B11* and *NLRP3*, which are in close proximity to cg14830002 and cg00112952, and correlated expression with the methylation levels in human samples and in HBECs [46]. In human samples, we did not detect significant correlation. We then exposed HBECs with 5-aza-2′-deoxycytidine, a DNAm inhibitor for 24, 48 and 72 h. We found that the expression of both *OR2B11* and *NLRP3* were significantly upregulated at 24 h ($p < 0.05$) (Figure 3) and a simultaneous reduction of methylation level at cg00112952, supporting the regulation of *OR2B11* and *NLRP3* expression by DNAm.

To gain insight into possible molecular mechanisms through which asthma-associated DNAm variation may regulate gene expression, we next examined the overlap between our verified asthma-associated CpG sites with experimental data monitoring the presence of open chromatin, histone marks and transcription factor binding events. Chromatic marks characteristic of enhancers (H3K4me1 and H3K27ac) and promoters (H3K27me3) were found at the cg00112952 and cg14830002 loci in multiple tissues/cell types including fetal lung. cg00112952 is also located in a region sensitive to DNase I treatment (indicating open chromatin) in multiple cell types, including the A549 lung derived cell line. Both sites also overlap binding sites for particular transcription factors. Specially, cg00112952 overlaps with ChIP-seq peaks for RAD21 and SOX2, while cg14830002 overlaps with peaks for RAD21, EP300, HNF4, GATA2, SMAD1, STAT3, C/EBPβ, MYC and MAX. Among these proteins, RAD21 and EP300 are chromatin regulators [47,48]; SOX2, GATA2, SMAD1, STAT3, C/EBPβ and MYC/MAX have all been implicated in lung development [49] and asthma pathogenesis [50–53], mostly through interaction with histone modifiers [54–59]. The *TET1*-associated CpG site cg23602092 overlaps with ChIP-seq peaks for RUNX1, FOXD2, RFX1, ZBTB7B, CDX2, C/EBP-α, MDB4 and MAX. In A549, a lung epithelial carcinoma cell line, cg23602092 is located within a region sensitive to DNase I treatment, and associated with active histone marks including H3K4me1/2/3 and H3K9ac, and H2A.Z (marks enhancer activity [60,61]). Collectively, these results support regulatory roles for cg00112952, cg14830002 and cg23602092 in gene expression.

Discussion

Here, we report asthma-associated DNAm variation in nasal DNA obtained from 29 child sibling pairs discordant for asthma in the ESS study. After accounting for known and unknown possible confounding factors, we identified six CpG sites whose methylation is nominally associated with childhood asthma. Three of these sites were replicated in independent cohorts and verified in 54 sibling pairs from the ESS study.

In addition to a site located in the *TET1* promoter we previously identified [17], two non-SNP CpG sites (cg00112952 and cg14830002) were identified within the promoter region of *OR2B11*. Both sites are also located

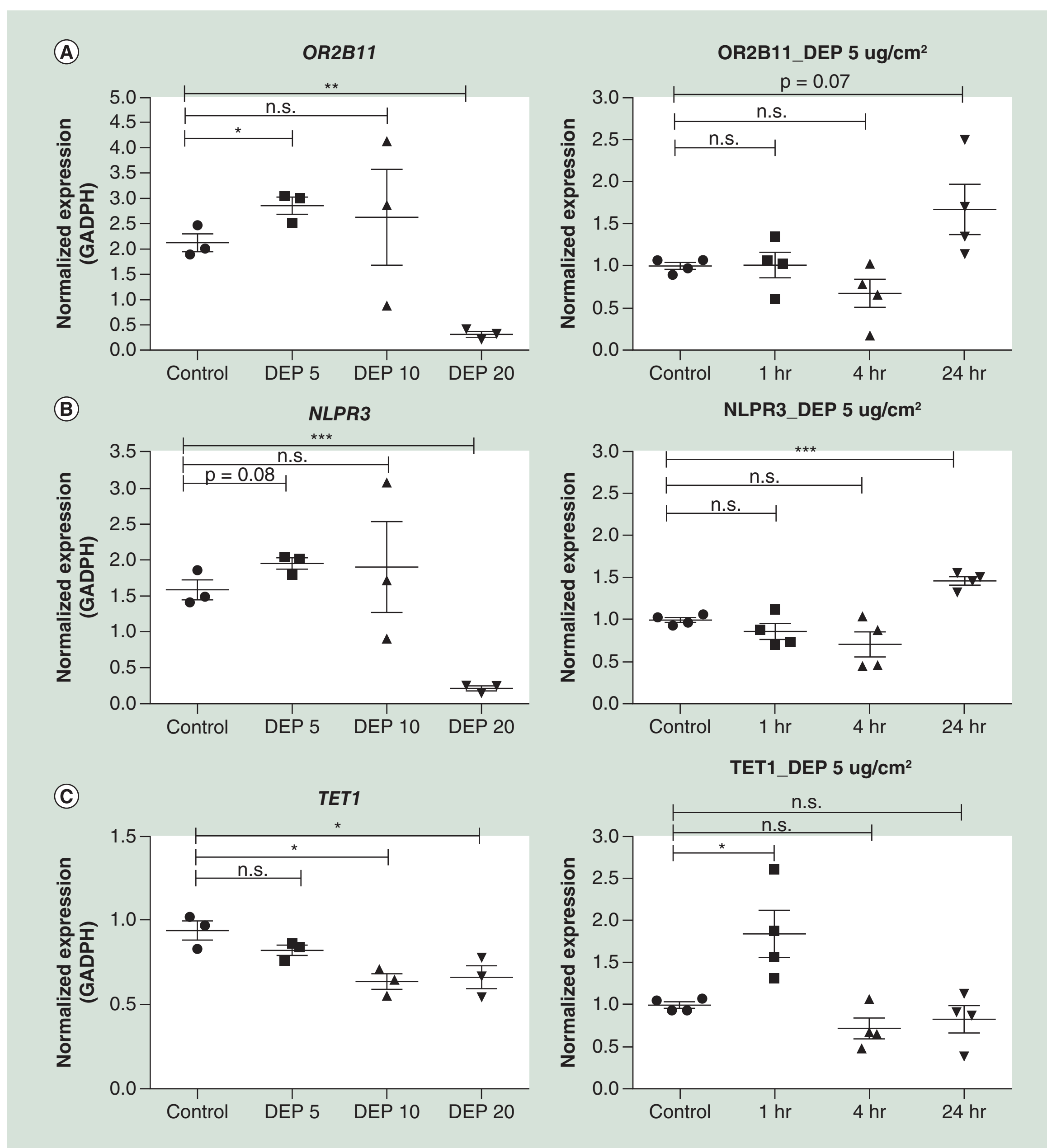

Figure 2. Dose- and time-specific response of *OR2B11*, *NLRP3* and *TET1* expression to diesel exhaust particle and house dust mite exposures in human bronchial epithelial cells. HBECs were exposed to indicated amount of DEP (5, 10 and 20 μg/cm^2) and HDM (25, 50 and 100 μg/ml), and expression of individual genes (*OR2B11* (**A**), *NLRP3* (**B**) and *TET1* (**C**)) were measured by RT-qPCR and normalized to *GAPDH* at 24 h postexposure. Mean values from three biological replicates are represented as the mean ± SD. Groups were compared using student's t-test.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
DEP: Diesel exhaust particle; HBEC: Human bronchial epithelial cell; HDM: House dust mite; n.s.: Nonsignificant; SD: Standard deviation.

approximately 4000 bp downstream the transcription end site of *NLRP3*, which encodes a protein that is part of the NALP3 inflammasome complex. This NALP3 complex functions as an upstream activator of NF-κB signaling, and plays a role in the regulation of inflammation, the immune response and apoptosis. It is required for asthma phenotypes in murine models [62], has elevated expression in neutrophilic asthma compared with controls and other asthma subtypes [63], and was recently reported to be a transcriptional regulator of Th2 differentiation [64]. NLRP3 was also activated by DEPs in *ex vivo* lung tissue explants [65] and in bronchoalveolar lavage fluid cells from mice [66].

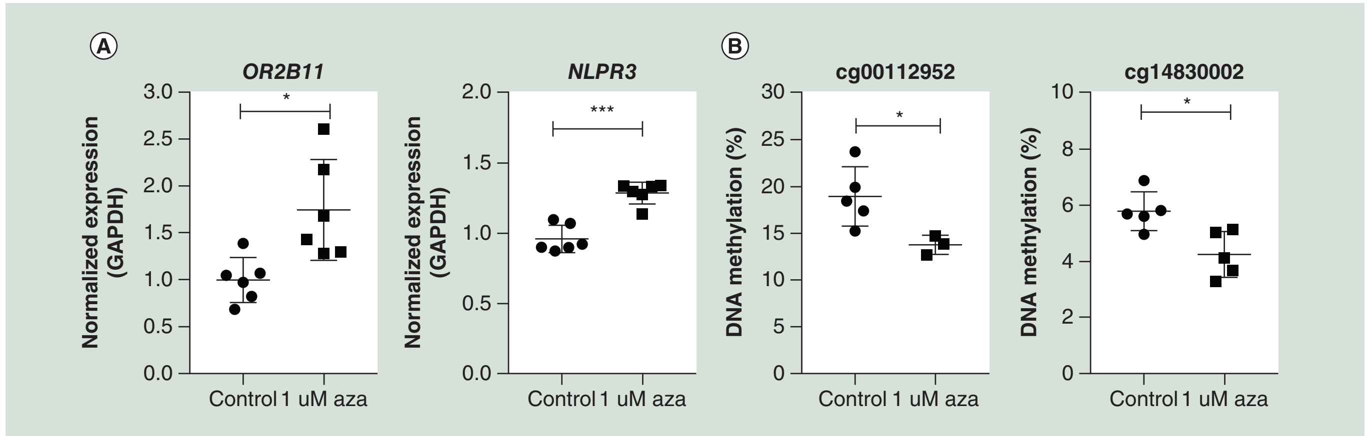

Figure 3. 5-aza treatment altered the expression of *OR2B11*, *NLRP3* and methylation of cg00112952. (A) Expression of *OR2B11* and *NLRP3* in HBEC treated with 1 μM 5-aza for 24 h. **(B)** DNAm levels of cg14830002 and cg00112952 in HBEC treated with 1 μM 5-aza for 24 h. Data represent two technical duplicates of three biological replicates and are shown as mean ± SD.
*p < 0.05; **p < 0.01; ***p < 0.001.
DNAm: DNA methylation; HBEC: Human bronchial epithelial cell; n.s.: Nonsignificant; SD: Standard deviation.

In agreement with these findings, we showed that in human bronchial epithelial cells exposed to various doses of DEP, mRNA levels of *NLRP3* were significantly altered within 24 h (Figure 2). This supports the involvement of epithelial *NLRP3* in response to asthma-related environmental exposure and in asthma development. Inhibition of DNAm using 5-aza-2′-deoxycytidine in airway epithelial cells resulted in increased expression of *OR2B11*, *NLRP3* and *TET1* (Figure 3 and [17]). Further experiments are needed to examine how methylation levels of cg00112952, cg14830002 and cg23602092 may alter gene expression through interactions with histone modifications and transcription factor binding.

One limitation of our discovery cohort was the small sample size. No sites were identified that reached the genome-wide significance though various methods to control family-wise error rate or FDR (false discovert rate) were attempted. To enhance power, nominal p-value at 0.05 was used as the p-value cutoff to identify candidate CpG sites. To reduce the risk of inflated type I errors, effect size was considered (difference in $\beta \geq 0.1$), as well as biological relevance. Aiming to identify the effects rather than accurately estimate them, the first genome-wide association studies often overestimate the effects and therefore need to be validated and replicated [67]. To further validate selected candidate CpG sites, an expended ESS cohort, and two other independent cohorts were also examined. We are able to replicate three CpG sites in two independent studies out of the six sites discovered from arrays. This highlights the usefulness of replication in compensation for potential inflated type I errors due to multiple testing. For the four sites that were not replicated, it could be due to a variety of factors, including false positives due to small sample size, different phenotype definitions used for both the controls and asthma (allergic vs nonallergic), different study designs (siblings vs unrelated cases and controls) or different analytical methods and cutoff. Using nasal mucosa samples from the ICAC study, Yang *et al.* identified 118 CpG sites solely based on p-values obtained from tests comparing DNAm between unrelated nonallergic healthy controls and allergic asthma [18]. For these 118 sites, four sites had p-value < 0.05 in our ESS study, but none were selected as significant candidates, as the largest difference in DNAm between control and asthma was 5.3% (located within the promoter of *CYP2E1*) and did not reach our effect size cutoff. In addition, when atopic asthmatics were compared with controls that were all nonallergic, the differences in DNAm could be due to both allergy and asthma. To specifically identify asthma-related genes, we included both allergic and nonallergic controls in our study. We also performed *post hoc* examination on association with allergy and asthma of identified sites (Table 3).

Similar to genetic studies, family and sibling-based study designs in epigenome-wide association studies have several advantages over studies using unrelated individuals [68]. First, sibling studies are robust in controlling for effects of population stratification. Second, genetic background and known or unknown environmental factors that may modify the epigenome (TRAP, SHS, diet, etc.) are controlled to a certain degree between cases and controls, which is difficult to achieve in studies using unrelated individuals. Behaviors related to age might affect

TRAP exposures and two siblings in the same household may have different TRAP exposures. Therefore, accurate assessment of personal exposure is desirable in future studies.

Conclusion

In summary, we identified three novel CpG sites (cg00112952, cg14830002 and cg23602092) whose DNAm levels were associated with childhood asthma. Of the three, cg14830002 was associated with both asthma and allergy, while the other two were not associated with allergy. Moreover, cg23602092 was associated with asthma symptom frequency among asthmatics. Two of three sites were also associated with current exposure to TRAP in nonasthmatics, and the expression of genes located near these sites were responsive to exposure of DEP and HDM in cultured airway cells and can be regulated by inhibition of DNAm. Additionally, these CpG sites are located at regions with active histone marks and open chromatin in many cell types, including airway epithelial cells. We conclude that these three CpG sites and their associated genes *OR2B11/NLRP3* and *TET1* may be novel candidates contributing to asthma pathogenesis and response to traffic pollution. Further, our results suggest that they could also be useful biomarkers to monitor asthma severity and exposure to traffic-related air pollution in children.

Summary points

- Methylation levels of cg00112952, cg14830002 and cg23602092 in nasal epithelial cells were associated with childhood asthma.
- Methylation levels of cg14830002 in nasal epithelial cells were associated with allergic symptoms in nonasthmatic controls.
- Methylation levels of cg23602092 were associated with asthma symptom frequency among asthmatics.
- Methylation levels of cg14830002 and cg23602092 were associated with current exposure to traffic-related air pollution in nonasthmatics.
- Exposure to diesel exhaust particle and house dust mite in cultured human bronchial epithelial cells altered the expression of *OR2B11*, *NLRP3* and *TET1,* genes located close to cg00112952, cg14830002 and cg23602092.
- Inhibition of DNA methylation altered the expression of *OR2B11*, *NLRP3* and *TET1*, genes located close to cg00112952, cg14830002 and cg23602092.
- Genomic locations near cg00112952, cg14830002 and cg23602092 are enriched in open chromatin, active histone marks and transcription factor binding sites.
- Collectively, our data identified DNA methylation variations associated with asthma and asthma-related environmental exposures and suggested possible mechanisms through which they may impact gene expression and contribute to disease.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at:
www.futuremedicine.com/doi/suppl/10.2217/epi-2017-0127

Authors' contributions

H Ji conceived and designed the experiments in discussion with X Zhang and JM Biagini Myers, performed pathway analysis; X Zhang performed Illumina bead array analysis and statistical analysis in discussion with H Ji; JD Burleson processed samples, performed locus-specific bisulfite pyrosequencing to measure DNAm and RT-qPCR to measure gene expression; JD Burleson, A Ulm and KS Bryan recruited children under the supervision of H Ji and JM Biagini Myers, managed Redcap database and performed sample processing; X Chen and MT Weirauch performed SNP annotation for 450K arrays and functional genomics analyses; MS Butsch Kovacic supported early study design and recruitment of unrelated, nonasthmatics in an underserved community; TA Baker supported recruitment of unrelated study participants from the underserved community; H Ji prepared the manuscript with the assistance of X Zhang, JM Biagini Myers, MT Weirauch and MS Butsch Kovacic. All authors read and approved the final version of manuscript before submission.

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Ethical conduct of research

Studies included in this manuscript were approved by the Institutional Review Board at Cincinnati Children's Hospital Medical Center (CCHMC).

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