

DNA Methylation Levels Associate with Race and Childhood Asthma Severity

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Abstract

Objective: Asthma is a common chronic childhood disease worldwide. Socioeconomic status, genetic predisposition and environmental factors contribute to its incidence and severity. A disproportionate number of children with asthma are economically disadvantaged and live in substandard housing with potential indoor environmental exposures such as cockroaches, dust

mites, rodents and molds. These exposures may manifest through epigenetic mechanisms that can lead to changes in relevant gene expression. We examined the association of global DNA methylation levels with socioeconomic status, asthma severity and race/ethnicity.

Methods: We measured global DNA methylation in peripheral blood of children with asthma enrolled in the Kansas City Safe and Healthy Homes Program. Inclusion criteria included residing in the same home for a minimum of 4 days per week and total family income of less than 80% of the Kansas City median family income. DNA methylation levels were quantified by an immunoassay that assessed the percentage of 5-methylcytosine.

Results and Conclusions: Our results indicate that overall, African American children had higher levels of global DNA methylation than children of other races/ethnicities ($p=0.029$). This difference was more pronounced when socioeconomic status and asthma severity were coupled with race/ethnicity ($p=0.042$) where low-income, African American children with persistent asthma had significantly elevated methylation levels relative to other races/ethnicities in the same context ($p=0.006$, Hedges $g=1.14$). Our study demonstrates a significant interaction effect among global DNA methylation levels, asthma severity, race/ethnicity, and socioeconomic status.

Introduction

Asthma is a common chronic childhood disease worldwide and represents a significant health problem and economic burden to families and the healthcare system [1]. Genetic predisposition, socioeconomic status, race/ethnicity and environmental factors contribute to asthma incidence [2]. Furthermore, it seems probable that each contributing factor interacts with other factors to guide prevalence and severity of the disease resulting in a complex spectrum of manifestations.

It is becoming clear that environmental factors can create heritable changes in gene expression that manifest as subtle alterations in phenotype and function. This process is referred to as epigenetic causation and recently has become a major point of investigation as a source of influence on disease severity and prevalence. Epigenetic mechanisms have been shown to influence the expression of genes involved in the inflammation that occurs in asthma [3]. In most cases studied thus far, epigenetic changes are mediated by altered DNA methylation, post-transcriptional histone modifications or elaboration of non-coding RNAs [4,5]. Of these, DNA methylation is the most extensively studied in asthma [6, 7].

A disproportionate number of children with asthma live in economically disadvantaged urban communities [8,9] and quality of housing influences asthma prevalence. Substandard housing is associated with increased indoor environmental exposures such as cockroaches, dust mites, rodents and mold, all of which can trigger asthma. Demographic profiles show that African American and Hispanic children are among the majority of children living in poorer housing conditions [10, 11]. Furthermore, asthma exacerbation and emergency department visits are highest among African descent groups [8,12]. From 2001 to 2010 African American children were twice as likely to have asthma as Caucasian children [13].

The Kansas City Safe and Healthy Homes Program (KCSHHP) was initiated to evaluate the impact of home remediation on asthma severity. We showed that simple low-cost interventions directed toward producing cleaner indoor air coupled with healthy home education, improved indoor air quality and health in asthmatic children [14]. The aim of the present study was to measure DNA methylation levels using cells taken from peripheral blood of children with asthma who were enrolled in the KCSHHP from 2008 to 2012. The intent was to identify parameters that can be used to guide remediation and monitor outcomes so that the most effective care can be offered and assessed in a timely and directed manner. Dynamic changes in DNA methylation in response to interventions have been documented. For example, changes in DNA methylation have been seen in adipose tissue from adult male subjects who underwent a six months exercise intervention [15] as well as in peripheral blood cells taken from obese adolescents after a 10-week weight loss intervention [16].

The present study represents an assessment of global DNA methylation levels in association with socioeconomic status, asthma severity and race/ethnicity in children with asthma.

Materials and Methods

Study population

Subjects were recruited during the interval between 2008 and 2012 to participate in the Kansas City Safe and Healthy Homes Program (KCSHHP). Inclusion criteria for participation included children between the ages of 2 and 17 yrs; physician confirmed diagnosis of asthma, chronic respiratory symptoms, chronic allergy symptoms or other chronic symptoms affected by the home environment; residing at the same home for a minimum of 4 days per week; living in

the same home for the last 6 months with plans to live in the same home for the next 12 months; and whose total family income was less than 80% of the Kansas City median family income of \$55,311. At a scheduled clinic visit severity of asthma was confirmed by a physician to be either “intermittent” or “persistent” based on the guidelines set forth by the National Asthma Education and Prevention Program. One of the guidelines was the frequency of use of a short-acting beta₂-agonist. Children classified as having intermittent asthma used the agonist $\leq 2x$ per week whereas children with persistent asthma used the agonist $> 2x$ per week. Venous blood was drawn for allergy IgE screening and the remaining blood was stored frozen. Ninety-nine families that were enrolled in the KCSHHP study gave consent for use of the blood sample for DNA analysis.

The Children’s Mercy Hospital Institutional Review Board approved this study. Written informed consent was obtained from parents/guardians and assent was obtained from the subject when applicable for use of the blood sample for DNA work.

DNA Extraction and Global DNA Methylation Assay

Approximately 3 – 5 mL of frozen peripheral blood was thawed at room temperature and DNA was extracted from the cells using the GeneCatcher kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The genomic DNA was quantified with the Nanodrop-1000™ spectrophotometer (ThermoFisher Scientific, Waltham, MA).

Global DNA methylation was assessed using the 5-mC DNA ELISA kit (Zymo Research, Irvine, CA) that is based on the detection and quantification of methylated DNA using a 5-methylcytosine antibody. The DNA was denatured for 5 min at 98°C prior to its use in the assay. A standard curve was established using methylated/demethylated *E. coli* DNA. All samples

including the standard curve were assayed in duplicate. To quantify the percent 5-methylcytosine the calculated % 5mC was multiplied by the fold difference between *E. coli* and human CpG density. According to the manufacturer the *E. coli* CpG density per genome length is 0.075 and human CpG density per genome length is 0.009. Thus, the fold difference between *E. coli* and human CpG density is 8.3.

Statistical Analyses

Initial statistical modeling considered data including age, gender, smoke exposure, steroid use, asthma severity, race/ethnicity and income level. Due to the categorical nature of the data (i.e. excluding age, all were binary variables), interaction effects for global measures of DNA methylation were analyzed using general linear models specifically multifactorial ANOVAs. Examination of significant interaction effects utilized pairwise comparisons through independent-samples *t* tests and ANOVA models with Bonferroni corrections. All variables were investigated for normality of distribution prior to any statistical analysis. The level of significance was set at $\alpha = 0.05$ and all analyses were performed using SPSS 22 (IBM Corporation, Armonk, NY). Statistically significant differences (p value) were also determined by *t*-test.

Results

Demographics

Table 1 illustrates the demographics of these study participants. Most of the children self-reported as African American (41%), followed by non-Hispanic Caucasian subjects (30%). Subjects who identified as more than one racial/ethnic group were grouped in the “other”

category. More male children (n=55) were consented than female children (n=44). Sixty-nine percent of the children lived in homes in which the total family income was less than 50% of the Kansas City median family income (MFI) of \$55,311.

When the subjects were grouped based on asthma severity and race/ethnicity, a disproportionate number of children with persistent asthma were African American (58%) relative to all other races/ethnicities (**Table 1**, X^2 , $p = 0.018$).

The majority of children 42%, among the lowest-income group were African American (**Table 1**). However, this was not considered to be a disproportionate grouping pattern (X^2 , $p = 0.729$) when the subjects were grouped according to socioeconomic status and race/ethnicity and compared to all other races/ethnicities. Furthermore, neither Hispanics nor Caucasians were disproportionately represented in this data set.

Global DNA Methylation

Initial statistical analyses found no significant main effects or interaction effects for age, gender, steroid use, or smoke exposure on global DNA methylation levels and these were excluded from the statistical models.

Race/Ethnicity

We measured the percentage of 5-methylcytosine as an indicator of global DNA methylation levels present in the peripheral blood of children with asthma. When we examined the pattern of global DNA methylation levels among the subjects we found that African American children (57.0 ± 2.7 , $p = 0.029$) had slight but significantly higher levels of global DNA methylation than children of other races/ethnicities (49.4 ± 2.1) (**Fig 1A**) whereas a similar analysis of Caucasian

(48.2 ± 3.0 vs 54.4 ± 2.0 , $p = 0.097$) and Hispanic (51.7 ± 3.9 vs 52.8 ± 1.9 , $p = 0.796$) children showed no significant difference (**Fig 1B** and **Fig 1C**, respectively).

Socioeconomic Status

Next we added the contributing factor of socioeconomic status, defined in Table 1, as <50% MFI or $\geq 50\%$ but <80% MFI. When we compared methylation levels of subjects in the lower income group (<50% MFI) a significant difference was seen between African American (60.0 ± 3.3) children and children of other races/ethnicities (48.5 ± 2.7) ($p = 0.009$) (**Fig 2A**). This difference was not observed when either Caucasian children (49.4 ± 4.0 vs 54.7 ± 2.6 , $p = 0.29$) (**Fig 2B**) or Hispanic children (49.8 ± 4.1 vs 54.6 ± 2.6 , $p = 0.33$) (**Fig 2C**) were compared separately with children of other races/ethnicities from this socioeconomic group. However, no differences were noted among African American (55.7 ± 3.2 vs 51.0 ± 2.5 , $p = 0.27$) or Caucasian (50.7 ± 3.2 vs 54.2 ± 2.8 , $p = 0.42$) children and children of other races/ethnicities in the upper income group ($\geq 50\%$ but < 80% MFI) (data not illustrated). An insufficient number of Hispanic children in this economic category ($n = 2$) were consented to allow analysis (Table 1).

Asthma Severity

Rates of adverse asthma outcomes such as emergency department visits and hospitalizations have been reported to be higher among children of African descent than Caucasian children [13]. When we examined the levels of global DNA methylation among the children with persistent asthma, African American children had significantly higher levels (58.23 ± 4.3 vs 44.72 ± 4.3 , $p = 0.039$) (**Fig 3A**) and Caucasian children had significantly lower levels (35.48 ± 5.9 vs 56.45 ± 3.6 , $p = 0.027$) (**Fig 3B**) compared to children of other races/ethnicities. No differences were

observed when Hispanic children were compared separately from children of other races/ethnicities (51.10 ± 6.5 vs 53.86 ± 4.1 , $p = 0.75$) (**Fig 3C**).

Interaction Effect

When African American children were compared to all other ethnicities together, the data revealed a significant interaction effect among global DNA methylation levels, asthma severity, race/ethnicity, and socioeconomic status ($p = 0.042$, observed power = 0.53). Follow-up analyses determined there was a significant income x ethnicity interaction ($p = 0.021$, observed power = 0.66) for children with persistent asthma. In children with persistent asthma from the lowest income families (<50% KC MFI), significantly higher levels of global DNA methylation were observed in African American children compared to children of other races/ethnicities ($p = 0.006$, 95% CI: AA = 53.6, 71.1; Other = 30.4, 52.2) (**Fig 4A**). However, the observed difference between ethnicities in the upper 50% income group was not statistically significant ($p = 0.235$, 95% CI: AA = 17.2, 59.0; Other = 36.0, 72.1) (**Fig 4A**). This interaction effect was not observed among children with intermittent asthma in either income group (<50% KC MFI: $p = 0.56$; $\geq 50\%$ KC MFI: $p = 0.28$) (**Fig 4B**). One suggestion from these observations is that asthma morbidity is multifactorial and dependent on genetic susceptibility, socioeconomic status and perhaps environmental exposures that were not accounted for in our data collection.

Gender

Gender specific differences in DNA methylation have been reported such that females have significantly lower DNA methylation levels than males [17-19]. When we examined global DNA methylation differences based on gender we did not see any significant difference ($p = 0.71$)

between females (51.8 ± 2.9) and males (53.1 ± 2.1). When we divided the subjects into two age groups (5 – 11yrs and 12 – 21yrs) (**Table 1**) there was no significant difference ($p = 0.29$) between females (56.0 ± 4.1 ; $n=22$) and males (51.1 ± 2.7 ; $n=34$) in the 5 – 11yr age group. However, although not significant ($p = 0.09$), males in the 12 – 21yr age group trended to higher DNA methylation levels (56.4 ± 3.2 ; $n=21$) than females (47.6 ± 4.0 ; $n=22$) in this age group (data not illustrated).

Discussion

Childhood asthma has increased dramatically during the last 2 to 3 decades with worldwide prevalence between 100 and 150 million sufferers. It poses a major health and economic burden disproportionately affecting minorities in inner-city communities. Black race, Puerto Rican ethnicity and low household income are major predictors of asthma exacerbation and emergency department visits for asthma [8, 13]. Children from low income families are likely to live in substandard housing and be exposed to indoor environmental allergens such as cockroaches, rodents, dust mites, and mold that could potentially trigger asthma. These environmental exposures may manifest at the cellular level through epigenetic mechanisms that can lead to rapid and persistent changes in gene expression. DNA methylation is the most widely examined such epigenetic mechanism thus far. In general, hypermethylation results in gene silencing and hypomethylation leads to active gene expression. Changes in DNA methylation profiles have been associated with development of disease. Reduced levels of global DNA methylation have been observed with cancer development [20]. Conversely, in inflammatory mediated diseases

such as Type I diabetes an overall increase in hypermethylation was seen in affected individuals [21].

The present study examined the association of global DNA methylation levels with socioeconomic status, asthma severity and race/ethnicity. Our results indicate that overall, African American children had significantly higher levels of global DNA methylation than children of other races/ethnicities. This difference was more pronounced when socioeconomic status and asthma severity were considered. The higher levels of DNA methylation are unlikely to be accounted for by race/ethnicity alone, especially since no differences were observed when we compared the children at the higher income level. Thus, a relationship was observed among global DNA methylation levels, race/ethnicity, socioeconomic status and asthma severity. Although at this level of analysis, specific gene regulation pathways cannot be discerned, overall levels of methylation reflect overall alterations in gene expression.

Recently, the impact of socioeconomic status on epigenetic mechanisms has been realized. A study from the UK examined global DNA methylation in an adult cohort that was categorized by health and socioeconomic status [22]. The authors observed that the percentage of global DNA methylation was significantly lower in subjects in the most deprived socioeconomic group compared to the least deprived or affluent group. A similar pattern of DNA methylation was reported in a candidate gene study where DNA methylation of pro-inflammatory genes was lower in healthy individuals with low socioeconomic status [23].

In adults, a few studies have examined the effect of race/ethnicity and gender on global DNA methylation. In two studies African American subjects had significantly lower levels of DNA

methylation than non-Hispanic Caucasians [17, 24] whereas in another study of 28 adult subjects no difference was found in global DNA methylation between races [25]. A potential limitation of the last study was the small sample size. On the basis of gender, women had significantly lower levels of global DNA methylation than men [17-19].

Race/ethnicity and gender differences in DNA methylation have also been reported in children. Similar to adults, lower levels of DNA methylation were seen in healthy African American compared to Caucasian newborns and boys had higher levels of DNA methylation than girls [26, 27]. In our study African American children with asthma had higher levels of global DNA methylation than asthmatic children of other races/ethnicities. It is likely that socioeconomic status in addition to asthma diagnosis impacted our observations since 74% of the African American children in our study were from the lower income group. Overall, we did not observe a difference in methylation levels based on gender in our population of children with asthma. However, when we divided the samples into two age groups (5 -11 yrs) and (12-21 yrs), we did observe higher methylation levels among the males than females in the 12-21 age group. Lower levels of methylation in females have been attributed to the inactive X-chromosome [28] as well as hormonal cycles. A significant drop in methylation was observed when a female derived breast cancer cell line was treated with estradiol [29]. Thus, the lower methylation levels that we observed in the female subjects in the older age group might be due to hormonal changes in this age group.

Although differences have been documented in methylation between children and adults suggesting that there is an age-related decrease in global methylation [30] the high levels of

DNA methylation observed among our African American subjects could not be accounted for solely by age. Environmental factors are likely to play a role. Several studies demonstrate epigenetic mechanisms driven by environmental factors [31]. These environmental factors include exposure to polycyclic aromatic hydrocarbons (PAHs) present in ambient air, automobile exhausts and dust, secondhand tobacco smoke (SHS), and particulate air pollution. Increased global DNA methylation was associated with increased exposure to PAHs in an adult non-smoking population [32]. Reduced global DNA methylation as well as differential methylation of 8 CpG loci was found in children who experienced in utero smoke exposure [33]. In adult monozygotic twins discordant for asthma and SHS exposure, patterns of CpG methylation in T cells were strongly implicated in disease [34]. In the asthmatic twin, increased CpG methylation resulted in decreased protein expression and impaired T cell function. In addition, asthmatic twins with recent SHS exposure had a significant increase in methylation of CpG sites compared to asthmatics with no SHS exposure. Decreased DNA methylation was also seen in adults exposed to ambient particulate pollutants [35]. Moreover, differential DNA methylation patterns were observed in inner city children with asthma compared to healthy control subjects [36]. Thus, our results suggest that it is possible that genetic predisposition may play a contributory role in environmentally-induced epigenetic changes.

The main limitation of the present study is the small sample size. Only 99 subjects consented to allow DNA extraction and analysis of their sample. A second limitation is the use of peripheral blood cells as our source of DNA. The use of peripheral blood has been shown by others to be a valid biomarker of asthma phenotypes [37, 38]. Nevertheless, differences in DNA methylation have been reported among different subsets of leukocytes [39]. Global DNA

methylation profiles in neutrophils that represent a large proportion of cells in blood did not correlate with other cell types present in peripheral blood. Also race has been linked to differences in leukocyte counts [40]. Africans mainly from sub-Saharan Africa had lower total white cell and neutrophil counts than Caucasians. Since our study showed that African American children with persistent asthma had higher levels of global DNA methylation and if their leukocyte counts were lower than Caucasian children then our assessment of DNA methylation levels may be an under estimate.

Conclusions

Our study is the first to demonstrate an association among global DNA methylation levels with asthma severity, race/ethnicity and socioeconomic status. Our work, when fully realized, is intended to provide a measure of success of environmental remediation under the assumption that removal of environmental stimuli will allow a dynamic change in methylation patterns and that this will be accompanied by less severe disease. Further analyses are underway as are more detailed examination of specific methylation profiles.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Table 1 Demographics

Number of Subjects	99	
Gender	No.	%
Male	55	55.5
Female	44	44.5
Age	No.	%
Female (5-11yr)	22	22.2
Female (12-21yr)	22	22.2
Male (5-11yr)	34	34.4
Male (12-21yr)	21	21.2
Race/Ethnicity	No.	%
African American (5-21yr)	41	41.4
Caucasians (6-21yr)	30	30.3
Hispanic (5-13yr)	21	21.2
Asian (18yr)	1	1.01
Other (3-16yr)	6	6.06
Intermittent Asthma	No.	%
African American	20	32
Caucasian	25	40
Hispanic	14	22
Other	4	6
Persistent Asthma	No.	%
African American	21	58
Caucasian	5	14
Hispanic	7	19
Other	3	8
Socioeconomic Status	No.	%
≥50% but <80% MFI	28	28.3
African American	10	36

Caucasian	12	43
Hispanic	2	7
Other	4	14
<50% MFI	69	69.7
African American	29	42
Caucasian	18	26
Hispanic	19	28
Other	3	4
Undeclared	2	2

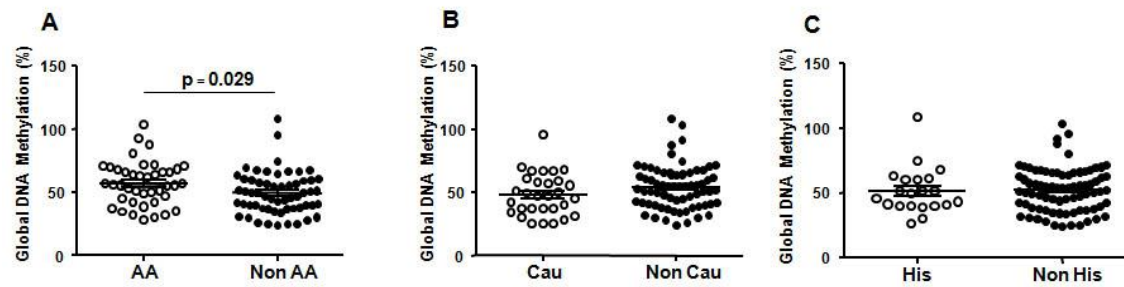


Figure 1.

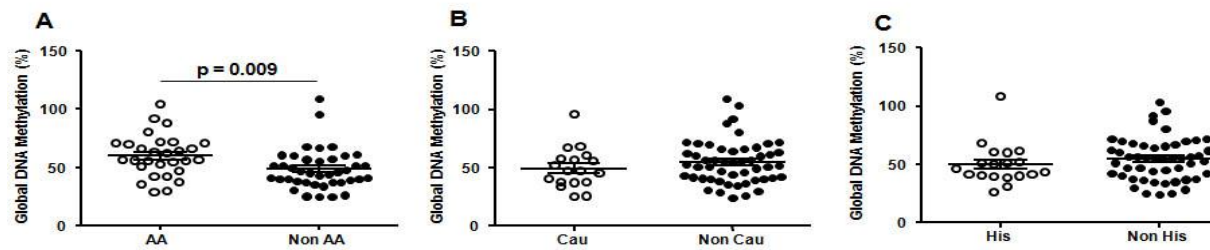


Figure 2.

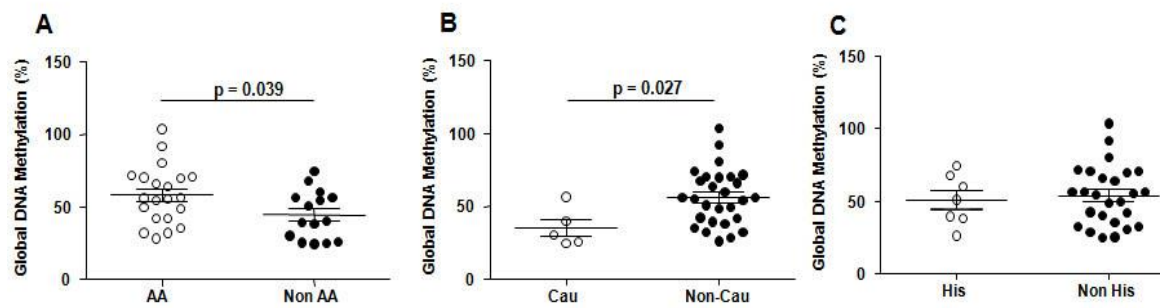


Figure 3.

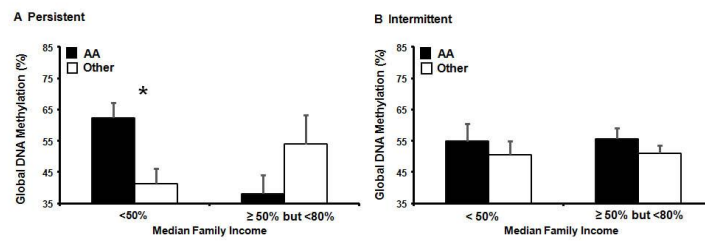


Figure 4.