DIFFERENTIAL DNA METHYLATION PROFILES OF INFANTS EXPOSED TO MATERNAL ASTHMA DURING PREGNANCY

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ABSTRACT

Background: Asthma is a complex disease that involves both genetic factors and environmental exposures. Aberrant epigenetic modifications, such as DNA methylation, may be important in asthma development. Fetal exposure to maternal asthma during critical periods of *in-utero* development may lead to epigenetic alterations that predispose infants to a greater risk of developing asthma themselves. We investigated alterations in the DNA methylation profile of peripheral blood from infants exposed to maternal asthma during pregnancy.

Methods: Peripheral blood was collected from 12 month old infants born to women with (n=25) and without (n =15) doctor diagnosed asthma during pregnancy. Genomic DNA was extracted, bisulfite converted, and hybridised to Infinium Methylation27 arrays (Illumina), containing over 27,000 CpGs from 14,495 genes. CpG loci in only autosomal genes were classified as differentially methylated at the 99% level ($p<0.01$, $|DiffScore| > 22$ and delta beta >0.06).

Results: There were 70 CpG loci, corresponding to 67 genes that were significantly differentially methylated. Twelve CpG loci (11 genes) showed greater than 10% comparative difference in DNA methylation, including hyper-methylated loci of *FAM181A*, *MRI1*, *PIWIL1*, *CHFR*, *DEFA1*, *MRPL28*, *AURKA* and hypo-methylated loci of *NALP1L5*, *MAP8KIP3*, *ACAT2* and *PM20D1* in the maternal asthma group. Methylation of *MAPK8IP3* was significantly negatively correlated with maternal blood eosinophils (r=-0.38; p=0.022), maternal eNO (r=-0.44; p=0.005), and maternal serum total IgE (r=-0.39, p=0.015). Methylation of *AURKA* negatively correlated with maternal haemoglobin (r=-0.43; p=0.008), as well as the infants height (r=-0.51; p<0.001) and weight (r=-0.36; p=0.021). Methylation level of *PM20D1* was lower in infants born to mothers with asthma on inhaled corticosteroid

treatment. Methylation of *PM20D1* was lower and *MRI1* was higher in infants born to atopic mothers without asthma.

Conclusions: Exposure to maternal asthma during pregnancy alters peripheral blood DNA methylation profile of infants which may act as risk factors for future asthma development.

INTRODUCTION

Asthma is a complex polygenic disease that results from genetic susceptibility and environmental exposure. This interaction promotes the chronic and heterogeneous inflammatory response and the airway hyperresponsiveness that characterise asthma. A key mechanism that underpins how environmental exposures modulate gene expression is via methylation of cytosine of the CpG dinucleotide (DNA methylation) in the gene-promoter regions, a well characterised and long-lasting epigenetic phenomenon. Therefore, these epigenetic alterations independently or together with genetic factors may underpin asthma susceptibility. This susceptibility occurs from conception since the intra-uterine microenvironment is pivotal in sustaining fetal development as well as re-shaping of the epigenome. Certain exposures during pregnancy such as adverse nutrition, smoking and maternal diseases are known to effect both fetal outcomes such as still births, fetal growth restriction and predisposition to variety of diseases [1-3]. Asthma is one of the most common medical illnesses to complicate pregnancy, with prevalence of 12% in Australia [4]. It is associated with both poor pregnancy outcomes [5-7] and with the development of childhood asthma. For example, maternal asthma has been found to be the strongest predictor of childhood asthma [8] and in addition, poor control of maternal asthma during pregnancy is associated with a further increased risk of childhood asthma [9].

The mechanisms by which maternal asthma contributes to these neonatal adversities include a direct effect of an altered cytokine environment induced by asthma, including reduced pro-inflammatory cytokine responses to immune challenge [10]. Other possibilities are that exposures associated with maternal asthma, such as the use of medications including corticosteroids and the effects of exacerbations during pregnancy may play a role.

Studies based on phenotypically measurable outcomes such as birth weight and infant wheezing therefore show that maternal asthma, its complications and its treatment during pregnancy can contribute to adverse outcomes for the infant. The intermediate stages that mediate these changes have not been defined. Maternal asthma likely induces modifications to the intra-uterine environment which could influence the developing epigenome of the infant, either causing immediate phenotypic manifestations, such as alterations to infant weight, or other silent epigenetic alterations that become active later in life, leading to increased disease susceptibility. Although it has been suspected that epigenetic changes may play a vital role in these effects, there have been no studies investigating epigenetic effects of maternal asthma during pregnancy.

Our objective was therefore to investigate alterations in the peripheral blood DNA methylation profile of twelve month old infants due to maternal asthma during pregnancy and relate these to phenotypic change in the infants, as well as exposure characteristics in the mother. We hypothesised that maternal asthma would result in characteristic changes in the DNA methylation profile in peripheral blood of infants.

METHODS

Study design and participants

The study population consisted of a cohort of infants (n= 40) born to women who participated in the Managing Asthma in Pregnancy Study [11] and a cohort study of viral infection in pregnancy, and were prospectively followed in infancy (the Growing Into Asthma birth cohort). Pregnant women with and without asthma were initially recruited between 12 and 20 weeks of gestation and had monthly clinical assessments of symptoms, lung function, and Fraction of exhaled nitric oxide (FeNO, Ecomedics, Switzerland) until delivery. Asthma diagnosis was confirmed by a respiratory physician (PGG) and women with current (past year) symptoms and/or medication use were enrolled.

Detailed clinical assessments were performed on infants at birth, 6 months and at 12 months of age by a respiratory paediatrician (JM). Additional information such as respiratory and allergy symptoms, growth and general health were obtained by a validated questionnaire [12] and by interviewing a parent/guardian. Mothers and their infants were excluded from our analysis if they were current smokers or had significant smoke exposure as determined by detection of urinary cotinine (NicAlert Urine Screen, Accutest, Encino, CA, USA). A sample of blood was collected from the infants at age of 12 months. The study was approved by the Hunter New England Health and University of Newcastle Human Research Ethics Committees.

DNA extraction and bisulphide conversion

Genomic DNA was purified from fresh frozen aliquots of whole blood (200ul) using the DNAEasy Mini Kit (Qiagen, Victoria, Australia), and quantified by Pico-Green assay (Life Technologies, Victoria, Australia) following manufacturer's recommendations. A Nano drop

2000 (Thermo Fisher scientific, Victoria, Australia) was used for assessment of DNA quality (the 260nm/280nm optical density ratio of 1.7-2.1 was deemed acceptable). In all, 500ng of DNA was treated with sodium bisulphide using the EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's recommended procedure adapted for Illumina's Infinium methylation analysis.

Genome-wide methylation assay

DNA methylation analysis was conducted using the Illumina Infinium Human Methylation27 Bead Chip (Illumina, San Diego, CA, USA). These arrays facilitate high-throughput processing and analysis of DNA methylation at genome-wide scale by interrogating over 27,000 highly informative CpG di-nucleotides, spanning 14495 genes. The assay is highly accurate and powerful in detecting the methylation status of queried CpG loci [13,14] and has extremely high reproducibility [13]. To assess DNA methylation, we used the standard Illumina protocols. In brief, the bisulphide converted samples were whole genome amplified and the amplified products were then fragmented by an endpoint enzymatic process. The fragmented DNA was purified and hybridised to Infinium Human Methylation27 arrays. During hybridization, the amplified and fragmented DNA samples anneal to locus specific DNA oligomers residing on the bead chips. Single base extension reaction, washing and staining were then carried out using a TECAN Te-Flow chamber. The stained arrays were assessed for fluorescence intensities at the methylated and unmethylated bead sites using Illumina BeadArray Reader (Illumina, San Diego, CA, USA).

Data analysis

Raw fluorescence data were processed using the Illumina methylation module (version 1.0.5) of the GenomeStudio™ software (version 1.0.2.20706). The β value was used to estimate the methylation level of the CpG locus by calculating the ratio of intensities between methylated

and unmethylated alleles (1>Average β>0 represents fully methylated to un-methylated alleles). A quality analysis was performed on each array in relation to bisulphide conversion efficiency, hybridisation efficiency and specificity, single base extension rate, target removal, staining for negative controls and staining for non-polymorphic probes. Prior to statistical analysis, the detection p value of 0.05 was used to eliminate CpGs that were poorly detected. In order to eliminate systematic differences between males and females, all CpGs residing on X and Y chromosomes were dropped from the analysis.

GenomeStudio software generates a DiffScore value to illustrate difference between groups, and is closely related to statistical p-values. The DiffScore, takes into account background noise and sample variability [15]. For a CpG locus to be differentially methylated it must satisfy two criteria, 1) Absolute DiffScore calculated using the Illumina custom model adjusted for multiple comparisons using the Benjamini and Hochberg false discovery rate must be greater than 22 (equivalent to adjusted $p < 0.01$), and 2) the degree of difference measured by the change in β value (Δβ) is greater than 0.06 (corresponding to an average of 6% or greater methylation difference). This statistical model bears the assumption that the methylation value β is normally distributed among biological replicates corresponding to a set of biological conditions. The outcome, DiffScore of a probe is computed as:

$$\text{DiffScore} = 10 \text{ sign } (\beta\text{asthma} - \beta\text{normal}) \log10p$$

$$\text{Delta}\beta = (\beta\text{asthma}, - \beta\text{normal})$$

Illumina custom error model and DiffScore has been described previously [16]. To investigate which biological functions of genes are significantly enriched, a gene ontology analysis was performed using PANTHER [17].

Further statistical analysis was performed using PRISM version 5 (GraphPad software, San Diego, CA, USA) and Stata 11 (StataCorp, TX, USA). Parametric and non-parametric data are presented as means (standard deviation) and medians (quartile 1, quartile 3) unless otherwise stated. Categorical data were compared using the χ^2 test or Fisher's exact test. For comparison of 2 continuous variables the 2-sample Student's t test was used, and for parametric data the Mann-Whitney U test was used. Multiple comparisons of continuous variables were analysed using one-way ANOVa with Bonferroni correction for parametric data, and Kruskal-Wallis test for non-parametric data. Associations of methylation levels of individual genes were tested using Pearson correlation for parametric data and Spearman correlation for non-parametric data. Associations were first investigated using a correlation matrix, following up the significant results using a scatter plot to visualise the distribution of the data. Statistical significance was defined as $p<0.05$.

RESULTS

Clinical features

The clinical characteristics of the infants and their mothers are detailed in Table 1. Although infants had normal anthropometry at birth , at 12 months, the average height of infants born to mothers with asthma was significantly lower than infants born to mothers without asthma. The mothers with asthma had elevated fractional exhaled nitric oxide (FeNO) as recorded at the first visit (gestation weeks 16-20) compared to mothers without asthma. More than half (64%, n= 14) of the asthmatics were taking inhaled corticosteroids (ICS), and 48% of women had one or more asthma exacerbations (defined by hospitalisation, emergency department visit, course of oral steroid or unscheduled doctor visit) during their pregnancy. A higher proportion of women were atopic in the maternal asthma group (80%) compared to only 40% of non-asthmatic women (Table 1). Significantly increased total IgE and reduced red cell count was observed in mothers with asthma, as well as a trend for lower maternal haemoglobin levels compared to mothers without asthma (Table 2).

Differential DNA methylation in peripheral blood of infants due to maternal asthma

The differential methylation status across 27,578 CpG dinucleotides was tested. Pre-processing identified 69 loci with poor detection and these were dropped from the analysis. Only autosomal loci were selected from the remaining and 26,475 loci were analysed for differential methylation. There were 159 CpG loci that were significantly differentially methylated (DiffScore >22, $p<0.01$) in the peripheral blood of infants born to mothers with asthma compared to mothers without asthma. Seventy of these differentially methylated CpG loci had a $\Delta\beta$ of greater than 0.06, and this corresponded to 68 genes. The 68 differentially methylated genes are listed in E-Table 1 in the supplementary material. Of these 70 CpG loci, 46 had increased methylation (hypermethylation) associated with maternal asthma, whilst 24

had a decreased methylation (hypomethylation) associated with maternal asthma. The most significantly differentially methylated genes with the greatest change in Δβ (>0.1 or 10% methylation) are listed in Table 3. Of those CpG loci presented in Table 3, 7 CpGs had increased methylation (hypermethylation) and 4 had decreased methylation (hypomethylation). *NAP1L5* is a paternally imprinted gene [18] and consequently its methylation status cannot be attributed to maternal factors. The distribution of the methylation for 8 of these loci is presented in Figure 1.

To investigate significantly enriched gene ontologies, the list of 68 differentially methylated genes were mapped to PANTHER biological processes, molecular functions and cellular components. Of the 68 genes, 61 were mapped to the PANTHER categories. There were 5 biological processes and 3 molecular functions that were significantly enriched ($p<0.02$ detection rate versus the whole genome), shown in Table 4.

Effects of asthma medication and atopy

The effects of maternal ICS use during pregnancy and maternal atopy on the methylation level of the differentially methylated genes (shown in Table 3) were further investigated. Methylation of the CpG locus of *PM20D1* was the only gene significantly associated with ICS treatment for maternal asthma during pregnancy ($p=0.039$; Figure 2A) and maternal atopy ($p=0.0031$; Figure 2B).

Clinical Associations

The relevance of the changes in peripheral blood DNA methylation of infants associated with maternal asthma during pregnancy was investigated regarding their relationship to clinical parameters and peripheral blood cell counts of the mother and the infant. Interestingly,

methylation of *MAPK8IP3* was significantly negatively correlated with maternal eosinophils (Figure 3A), maternal FeNO (Figure 3B), maternal serum total IgE (Figure 3C). However the methylation of this locus not associated with infant's blood eosinophil count (r=0.04, p=0.82). Methylation of *PM20D1* was also negatively correlated with maternal serum total IgE [cg14893161: r=-0.39; p=0.01, cg14159672: r=-0.42, p=0.009]. The level of *AURKA* methylation negatively correlated with infants height (Figure 3D) and weight (Figure 3E) at 12 months and with maternal haemoglobin (Figure 3F). Methylation level of *DEFA1* was associated with infants peripheral blood neutrophils [r=-0.43, p=0.01] and lymphocytes [r=-0.44, p=0.01]. *FAM181A* methylation was associated with maternal ACQ score [r=-0.47, p=0.02].

DISCUSSION

This study demonstrates for the first time, that maternal asthma during pregnancy is associated with epigenetic alterations in the peripheral blood of infants. There were 68 genes that were significantly differentially methylated. These genes were overenriched in biological processes related to lipid, steroid and fatty acid metabolism and molecular functions related to enzyme inhibitor/regulator activities. There were 12 CpG loci (11 genes) that were prominent with greater than 10% change in methylation status. These loci were *FAM181A*, *MRI1*, *PIWIL1*, *CHFR*, *DEFA1*, MRPL28 and AURKA, which had increased methylation due to asthma, and *NALP1L5*, *MAP8KIP3*, *ACAT2* and *PM20D1* which had decreased methylation in asthma.

Many of the above genes involve in key regulatory pathways concerning developmental, metabolic and inflammatory processes. In particular, we have previously reported the alterations in *DEFA1* expression in phenotypes of asthma [19]. Located in chromosome 8p23.1, *DEFA1* plays a role in phagocyte-mediated host defence [20]. The gene is expressed predominantly in blood and bone marrow. The degree of methylation of *DEFA1* in this study also correlated with infants' lymphocyte counts and neutrophil counts. Since DNA methylation is an important epigenetic regulator of gene expression, generally increased methylation correlates with suppressed transcription. In blood, neutrophils are the major source of *DEFA1* and our previous study has shown upregulated gene expression of *DEFA1* in neutrophilic asthma compared to eosinophilic asthma in adults [19]. The current study has found that *DEFA1* has an increased methylation in blood of infants in the maternal asthma group, which would be expected to relate to suppression of *DEFA1* gene expression. This is

likely to be related to an allergic Th2 microenvironment of the mother, however further investigation is needed regarding the role of defensins in asthma.

Certain genes from our list carry important roles in multiple cellular and inflammatory pathways. In particular *MAPK8IP3* gene, a member of MAPK pathway may be important in allergic diseases such as asthma. Located in chromosome 16p13.3, *MAPK8IP3* is an integral component of MAPK cascade, regulating MAPK/JNK signalling. There is evidence that asthma T helper 2 phenotype (allergic asthma) was associated with MAPK pathway [21]. Existing literature suggests MAPK signalling pathways are important in the differentiation of CD4 and CD8 T cells during thymopoiesis [21,22], differentiation of eosinophils [23]. In addition it plays a role in regulating the functioning of T cells, eosinophils, mast cells and dendritic cells in the periphery (reviewed in [24]). Interestingly, we observed not only hypo-methylation of *MAP8KIP3* locus in the infants but also the level of methylation in *MAPK8IP3* was negatively correlated with maternal blood eosinophils, FeNO and Total IgE, indicating this methylation difference may also result from maternal epigenetic modification inherited by the infant. However we did not observe a correlation of *MAP8KIP3* locus methylation with infants' blood eosinophil count. Although biological outcomes of the hypo-methylation of this locus in infants need further investigation, the differential methylation together with correlation of the methylation with maternal clinical parameters suggests exposure effects of maternal asthma.

The locus for *CHFR* was also hypermethylated in asthma. Located in chromosome 12q24.33, *CHFR* is a ubiquitin ligase, involved in delaying mitosis (metaphase) in response to mitotic stress and in ubiquitanilation of other proteins such as *AURKA*, *PLK1* [25] and histone deacetylase 1 (*HDAC1*) [26], leading to their degradation. Recent studies have shown that *CHFR* is a negative regulator of the nuclear factor-κB (NF-κB) pathway [27]. An important effect of this down-regulation of (NF-κB) is the concurrent down-regulation of interleukin

(IL)-8 [27]. *CHFR* is also known to interact with *HDAC1*, causing polyubiquitylation and down regulation of HDAC activity [26]. *CHFR* gene methylation was recently associated with persistent wheezing in children [28] further supporting a potential role of this gene in the development of respiratory diseases. Together, these findings suggest that regulation of this gene by gene suppression may lead to overactivity of innate cytokine pathways and development of wheezing in infants, which may predispose to the development of asthma.

The locus for *AURKA* was also significantly hypermethylated in asthma. The gene is located in chromosome 20q13, has similar functions to *CHFR*, involving many aspects of mitosis and expressed in many tissues throughout development. However *AURKA* is a serine/threonine kinase capable of phosphorylating many proteins including its own inhibitors. We report methylation of *AURKA* was correlated with infants' height, weight and average maternal haemoglobin levels during pregnancy. This would indicate that the methylation status of *AURKA* may be associated with maternal hypoxia (haemoglobin levels) and play a role in the infants' growth and development.

The hyper methylated CpG locus of *FAM181A* was among the most prominent with 16% of methylation difference in the group comparison. Localised within chromosome 14q32.12, the exact function of this gene is yet to be unveiled. A recent study indicated differential methylation of this gene in cord blood was due to maternal micronutrient supplementation [29]. Also the authors noted that in cord blood this gene was differentially methylated in female infants [29]. Our study is the first to identify changes in methylation of *FAM181A* with maternal asthma, and its correlation with maternal ACQ score during pregnancy further indicates its potential importance in asthma.

The locus for PIWIL1 was also hypermethylated in asthma. This gene is located in chromosome 12q24.33, and functionally attributed to many aspects of meiosis [30]. Although

minimally expressed in normal tissues, over-expression of *PIWIL1* has been detected in many tumour types [31-33]. Increased expression of *PIWIL1* has been reported in asthmatic airway epithelium [34]. Expression of *PIWIL1* is regulated by RAS associated domain family (RASSF1C) which also regulates the MEK-ERK1/2 pathway [35], known to be important in the pathogenesis of asthma [36,37]. We observed hyper-methylation of this locus suggesting possible down regulated expression, in contrast to the up regulation reported in the epithelium, which may relate to the tissues sampled (i.e. blood vs airway epithelium) or the influence of developmental changes on the relationship between this gene and asthma.

The locus of *ACAT2* was also hypomethylated in our cohort of infants in association with maternal asthma. Located in chromosome 6q25.3, the product of this gene is an enzyme (acetoacetyl-CoA acetyltransferase 2) involved in lipid metabolism [38]. Although it had not been previously associated with asthma, the gene has been detected in blood monocytes, playing an important role in foam cell formation due to cholesterol accumulation in macrophages [39]. Foamy alveolar macrophages exhibit an increased pro-inflammatory function and have been associated with airway inflammation.

Considering functional aspects of the differentially methylated genes, many are known to play direct or indirect roles that are associated with immune and inflammatory processes. However functional aspects some others including *PM20D1*, *MRI1* and *MRPL28* are yet to be fully investigated. Despite this, the differential methylation of *PM20D1, a* carboxypeptidase PM20D1 precursor, is associated with maternal asthma, maternal medication use (ICS), maternal atopic status and association with maternal serum IgE may indicate an involvement of this gene in allergy and asthma, and may also indicate maternal epigenetic modification inherited by the infant. The protein is expressed in blood plasma, suggesting a probable secretion from blood cells.

Although this is the first study of its kind investigating changes to the DNA methylation profiles of 12 month old infants born to mothers with or without asthma, it has a number of limitations. The sample size of 40 infants may have limited our power in detecting significant methylation differences and clinical associations with DNA methylation levels. Future studies should follow up our findings in larger cohorts. Outcomes were assessed at 12 months and attributed to effects in-utero. Although most of the epigenome is re-shaped prior to birth, there are possibilities of post-natal alterations, as well as potential inheritance of differentially methylated genes from the mother [40], which warrant further investigation. We assumed maternal asthma was the main effect modifier; however residual or uncontrolled confounding cannot be completely excluded. These may include maternal nutrition during pregnancy, life style, and infant breastfeeding. We used whole blood, and since each cell type has a characteristic epigenome, we essentially assessed a mixture. We have only assessed autosomal loci because of systemic differences between genders. This approach is largely suitable given the small sample size however there may be some DNA methylation differences in autosomes due to gender [41]. Functional validation of the differentially methylated genes discovered in this study will be important in understanding their biological importance. While independent replications that address our limitations are needed, the current findings may have important and novel basis for understanding effects of maternal asthma towards the epigenome of infants.

In summary, we have identified 10 genes in which methylation statuses in infants' whole blood had significant association with maternal asthma during pregnancy. Our findings together with existing literature indicate the potential importance of these genes in the development of asthma. Follow up studies will be able to ascertain whether the methylation status of these genes are associated with the development of asthma in these infants, providing further clues to the biological mechanisms behind asthma development.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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