Epigenetic Markers for Transplacental Exposure to Airborne Polycyclic Aromatic Hydrocarbons (PAHs) and Childhood Asthma

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Epigenetic and Genetic interactions influence inter-individual disease risk

Environmental Factors

Epigenetics
(can be modified after conception)
Quantitative, sensitive to environmental stimuli, age-dependent

Genetic polymorphisms
(born with)

Inter-individual variability in disease risks

Modified from Miller and Ho 2008
Epigenetic changes are reversible, heritable modifications that do not involve alterations in the primary DNA sequence.

Three distinct and intertwined mechanisms:
- Non-coding RNAs, DNA methylation, and Histone modification

These processes affect transcript stability, DNA folding, nucleosome positioning and chromatin compaction.

Singularly or conjointly, they determine whether a gene is silenced or activated.
Epigenetics, environment and development

Environmental exposure

Maternal Factors

Diet and Life style

Gametes → Zygote → Embryo → Fetus → Baby → Adolescent → Adult → Elderly

Developmental epigenetic reprogramming

Somatic epimutation

Germline epimutation

Genome-wide Demethylation

Dysregulation of epigenetic processes → Disease development

Modified from Foley et al., 2008
Epigenetic epidemiology-key points

Epigenetic epidemiology studies show how the *inter-individual epigenetic variation affects disease risk.*

Sample selection

Statistical analysis

Study designs-DNA methylation

Use of *in vitro* and *in vivo* data
Airborne PAHs, Epigenetics, Asthma

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Airborne PAHs (common traffic-related air pollutants)

Transplacental exposure

Epigenetic reprogramming

Childhood asthma
Or airway inflammation
PAHs and Asthma

➢ Asthma is the most common chronic childhood disease and its risk may be strongly influenced by prenatal events (Selgrade et al., 2006).

➢ In our longitudinal cohort study of children residing in urban low-income and minority communities of New York City, the asthma rate exceeds 25% and is among the highest in the nation (Perera, Miller et al., 2003, 2004, 2006).

➢ We showed that in utero exposure to the common traffic-related air pollutants, polycyclic aromatic hydrocarbons (PAHs), is a risk factor for development of childhood asthma (Miller et al., 2004).
Study Population

Monitor PAH exposure: Personal prenatal air monitoring was conducted for two consecutive days during the 3rd trimester of pregnancy.

Asthma Status: Based on parental report of a doctor’s diagnosis of asthma or probable asthma before the age of five, children were classified as “asthmatic” or “non-asthmatic.” (“with or without a parental report of asthma”).
## Study Population

<table>
<thead>
<tr>
<th>Category</th>
<th>Subcategory</th>
<th>Full cohort (N=729)</th>
<th>Study sample (N=56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal ages (yrs)(mean ±SD)</td>
<td></td>
<td>25.1 ± 4.9</td>
<td>25.4 ± 4.6</td>
</tr>
<tr>
<td>Baby's sex (%)</td>
<td>Male</td>
<td>48.3</td>
<td>42.9</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>51.7</td>
<td>57.1</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td>Dominican</td>
<td>63.5</td>
<td>46.4</td>
</tr>
<tr>
<td></td>
<td>African American</td>
<td>36.5</td>
<td>53.6</td>
</tr>
<tr>
<td>Median total PAH (ng/m³)</td>
<td></td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Probable asthmatic up to 5 years (%)</td>
<td></td>
<td>27.2</td>
<td>26.8</td>
</tr>
</tbody>
</table>
- Level and pattern of epigenetic marks is **tissue-specific, cell-type specific** and may **vary over time**.

- **A wide range of samples**, if feasible, have to be collected and the **non-invasive sample collection** is recommended.
-Sample Selection-

- Maternal and/or umbilical cord blood sample at delivery were collected. DNA (100ng-500ng) was extracted from cord blood white blood cells (UCWBCs).

- UCWBC provide a reasonable surrogate for our target organ/tissue (lung/immune cells) because they contain stem cells that can populate in the lungs in later life and also provide a rich source of T cells which are important producers of cytokines and other asthma mediators.

- The matched fetal placental tissues (FPTs) provides ample tissue for obtaining fetal RNA to verify correlative changes in gene expression.

Columbia Center for Children’s Environmental Health (CCCEH)
- **DNA methylation** is the addition of a methyl group derived from S-adenosyl-L-methionine to the **fifth carbon of the cytosine ring** to form the fifth base 5-methyl cytosine.

- It occurs predominantly in cytosines located at 5’ of guanines, known as CpG dinucleotides (CpGs). These CpGs **cluster as CpG islands (CGIs)** in 1-2% of the genome.
- Few hundred ng levels of DNA are sufficient for DNA methylation studies.

- Percent methylation within a population of alleles can be measured with specific methylation site analyses or in a high-throughput manner.

- DNA methylation pattern is cell-type specific, gene specific and may change over time.

- Epigenetic regulatory regions have to be characterized and validated at all stages for all samples.
Workflow of DNA methylation studies

**Isolation of DNA from UCWBC samples**
(*n=10; PAH below cohort median (2.3 ng/m3); n=10; PAH above cohort median)

Methylation profiling tools
(i.e. Methylation Sensitive Restriction Fingerprinting)

DNA extracted from gel, re-amplified by PCR and subcloned into vector

Sequencing / BLAST / BLAT search

Promoter / CpG island search

Data confirmation

**Gene expression**
Real-Time PCR

**DNA methylation status**
- Bisulfite Sequencing
- Methylation-Specific PCR (MSPCR)
Using MSRF, we have analyzed UCWBCs from an initial sample of 20 CCCEH cohort children*.

(*n=10; PAH below cohort median (2.3 ng/m3); n=10; PAH above cohort median)
Validation of an association between CGI methylation status by bisulfite sequencing and PAH exposure level in the same n=14 UCWBC DNA samples – association was confirmed for all 6 candidate genes

Evaluation of concordance between degree of CGI methylation in UCWBC and level of transcript expression in n=14 matched FPT samples –

Choose potential epigenetic marker(s) and
test on another 56 samples in the cohort
Six MSRF candidates chosen for validation

A. **ACSL3**
   - CGI (1058bp)

B. **DUSP22**
   - CGI (927bp)

C. **RAD21**
   - CGI 1 (704bp)
   - CGI 2 (272bp)

D. **SCD5**
   - CGI (1108bp)

E. **SFMBT2**
   - CGI 1 (335bp)
   - CGI 2 (567bp)
   - CGI 3 (229bp)

F. **WWOX**
   - CGI (955bp)
# Methylation status and gene expression

14 UCWBC DNA and their fetal placental tissues (FPT) RNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>ACSL3 PAH level</th>
<th>ACSL3 Overall Met%</th>
<th>RAD 21 Overall Met%</th>
<th>DUSP22 Overall Met%</th>
<th>SCD5 Overall Met%</th>
<th>SFMBT2 Overall Met%</th>
<th>WWOX Overall Met%</th>
</tr>
</thead>
<tbody>
<tr>
<td>#813</td>
<td>1.71</td>
<td>42</td>
<td>46</td>
<td>2.22</td>
<td>52</td>
<td>26.72</td>
<td>0.46</td>
</tr>
<tr>
<td>#826</td>
<td>1.80</td>
<td>45</td>
<td>56</td>
<td>0.58</td>
<td>42</td>
<td>9.31</td>
<td>0.71</td>
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<tr>
<td>#926</td>
<td>1.50</td>
<td>48</td>
<td>35</td>
<td>6.36</td>
<td>35</td>
<td>6984.8</td>
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<td>#954</td>
<td>1.15</td>
<td>35</td>
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<td>0.66</td>
<td>36</td>
<td>12.70</td>
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<td>#1066</td>
<td>1.22</td>
<td>67</td>
<td>45</td>
<td>0.79</td>
<td>36</td>
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<td>#1090</td>
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<td>35</td>
<td>1.19</td>
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<td>13.45</td>
<td>1.41</td>
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<td>#784</td>
<td>34.48</td>
<td>85</td>
<td>80</td>
<td>1.33</td>
<td>80</td>
<td>30.25</td>
<td>1.41</td>
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<td>#831</td>
<td>2.41</td>
<td>81</td>
<td>90</td>
<td>2.18</td>
<td>85</td>
<td>5.15</td>
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<tr>
<td>#876</td>
<td>2.65</td>
<td>94</td>
<td>85</td>
<td>759.55</td>
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<td>#901</td>
<td>2.82</td>
<td>95</td>
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<tr>
<td>#946</td>
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<td>95</td>
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<td>#1079</td>
<td>3.66</td>
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<td>12.95</td>
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<tr>
<td>#1107</td>
<td>2.76</td>
<td>93</td>
<td>78</td>
<td>0.34</td>
<td>78</td>
<td>2.47</td>
<td>0.17</td>
</tr>
</tbody>
</table>

RER: Relative Gene Expression Ratio; Overall Met%: Promoter methylation percent

Hypermethylated at high PAH

Hypomethylated at high PAH
Evaluation of concordance between degree of CGI methylation in UCWBC and level of transcript expression in FPT

14 UCWBC DNA and their fetal placental tissues (FPT) RNA

Statistical Analysis

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>tau*</th>
<th>Confidence Interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACSL3</td>
<td>-0.45</td>
<td>[-0.76, -0.15]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>RAD21</td>
<td>-0.30</td>
<td>[-0.70, 0.09]</td>
<td>0.13</td>
</tr>
<tr>
<td>DUSP22</td>
<td>-0.31</td>
<td>[-0.66, 0.05]</td>
<td>0.09</td>
</tr>
<tr>
<td>SCD5</td>
<td>-0.11</td>
<td>[-0.49, 0.26]</td>
<td>0.55</td>
</tr>
<tr>
<td>SFMBT2</td>
<td>-0.23</td>
<td>[-0.57, 0.10]</td>
<td>0.18</td>
</tr>
<tr>
<td>WWOX</td>
<td>+0.22</td>
<td>[-0.11, 0.55]</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*The Kendall coefficient of concordance (tau) measures the strength of relationship between gene methylation and gene expression. Negative sign indicates inverse relationship.
It plays an essential role in fatty acid metabolism and is expressed in lung and thymic tissue.

Exposure to the PAH benzo(a)pyrene increased the expression of acyl-CoA oxidase, catalase and glutathione peroxidase (Orbea et al., 2002).

Interestingly, ACSL3 is located in 2q36.1 which has recently been shown to be associated with regions of the asthma susceptibility loci, in specific populations (Bouzigon et al., 2007; Choudhry et al., 2008).
Choosing ACSL3 as target

Validation of an association between CGI methylation status by bisulfite sequencing and PAH exposure level in the same n=14 UCWBC DNA samples – association was confirmed for all 6 candidate genes

Evaluation of concordance between degree of CGI methylation in UCWBC and level of transcript expression in n=14 matched FPT samples – the highest concordance gene was ACSL3

Establishment of an association between ACSL3 CGI methylation status, PAH level and childhood asthma in the 56 participants
ACSL3 promoter hypermethylated at high PAH
(Assayed by Bisulfite Sequencing)

Low PAH
No Asthma
Overall Met%: 68.3±2.1

High PAH
No Asthma
Overall Met%: 89.1±4.0*

Unmet
Met

ID#  PAH
755, 0.85
813, 1.71
826, 1.80
886, 1.72
1066, 1.22
70,  6.61
78,  2.43
104, 2.66
120, 4.87
946, 2.84
ACSL3 promoter methylation, PAH and asthma

Methylation-Specific PCR
- Standard epidemiology analytic tools can be applied to examine the association between the outcome and epigenetic factors and the interaction between the environmental factors and epigenetic factors.

- Several important covariates and potential confounders have to be concerned. Statistics algorithms (eg. false discovery rate) should be applied to identify as many true association as possible and minimize the overall portion of false-positive.
ACSL3 methylation is strongly associated with the levels of PAH exposure

56 samples from the cohort

Area under curve=0.818
Optimum cut-off value for PAH=2.41
Sensitivity=75%; Specificity=82%

Odds ratio: 13.8 (95% CI’s:3.8, 50.2)
ACSL3 methylation is associated with asthma at age 5

<table>
<thead>
<tr>
<th>ACSL3 5’CGI Methylation Status</th>
<th>Methylated</th>
<th>Unmethylated</th>
<th>All</th>
<th>Odds Ratios (95% CI’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma (% Yes with asthma)³</td>
<td>11/28 (39%)</td>
<td>4/28 (14%)</td>
<td>15/56 (27%)</td>
<td>3.9 (1.1, 14.3)</td>
</tr>
<tr>
<td>Median PAH exposure with in each group² (Min, Max)</td>
<td>3.39 ng/m³ (1.11, 34.48)</td>
<td>1.7 ng/m³ (0.49, 3.33)</td>
<td>2.26 ng/m³ (0.49, 34.48)</td>
<td></td>
</tr>
</tbody>
</table>
Exposure of H1299 to BaP increased methylation of ACSL3 promoter and lowered gene expression.
Animal models or cell line models can show how *environmental influences* can cause persistent changes in epigenetic gene regulation.

They are used to *discover the epigenetic signatures* directly associated with various environmental factors.
Conclusions

✓ Over 30 DNA sequences were identified whose methylation status was dependent on the level of maternal PAH exposure by using Methylation Sensitive Restriction Fingerprinting on 20 cord blood white blood cells samples.

✓ Methylation of the ACSL3 5’-CGI was found to be significantly associated with maternal airborne PAH when exposure exceeding 2.41 ng/m3 (OR=13.8; \( p<0.001 \); sensitivity=75%; specificity=82%) and with a parental report of asthma symptoms in children prior to age 5 (OR=3.9; \( p<0.05 \)).

✓ Thus, if validated, methylated ACSL3 5’CGI in UCWBC DNA may be a surrogate endpoint for assessing transplacental PAH exposure and/or predicting childhood asthma risk.

✓ This proof-of-principle study provides a new blueprint for the discovery of epigenetic biomarkers relevant to other exposure assessments and/or investigations of exposure-disease relationships in birth cohorts.
Future Prospects

- Can the markers be validated in other independent cohort studies?
- Can we identify a specific **signature for asthma**?
  - **Use of High-throughput platform for discovery**: Methylation Microarray, ChIP-sequencing, Transcriptome Profiling
  - **Use of High-throughput platform for validation**: MassARRAY, MethyLight

- Can the markers predict asthma caused by other risk factors, e.g. ozone, tobacco smoke, allergens?

**Characteristics of good biomarkers:**
- **Sensitive**
- **Specific**
- **Early stage indicator**

- How do polymorphisms in asthma-related genes interact with epigenetic mechanisms to confer higher susceptibility to environmental influences?
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