The Use of Proteomics in Elucidating Mechanisms of Predisposition for Mammary Cancer and Biomarkers of Effect

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Overarching goal: To determine the effects of perinatal exposure to environmental chemicals predisposing for mammary cancer.
BPA sport bottles

Nalgene Baby Bottle

Painting on BPA

Lining of soda and food cans

Office BPA H₂O
Oral Postnatal (Prepubertal) Bisphenol A Exposure

Lactating female Sprague Dawley CD rats are treated orally on days 2-20 post-partum with 25 µg or 250 µg BPA/kg body weight or an equivalent volume of sesame oil.

At day 50 postpartum female offspring are treated orally with 30 mg dimethylbenz(a)anthracene (DMBA)/kg BW to induce mammary tumors.

Palpate for mammary tumors
Necropsy at 180 days post DMBA
Histopathology carried out

Important time line: No exposure to BPA after weaning
DMBA Induced Mammary Tumors in Rats Exposed Prepubertally to Bisphenol A

Lactating female Sprague Dawley CD rats were fed 25 mg genistein or 250 mg genistein/kg diet from birth until time of weaning, hence the offspring receive genistein from the mother’s milk. (Offspring received genistein only during lactation.)

50 Day old female offspring received 80 mg DMBA/kg BW to induce mammary tumors.
DMBA Induced Mammary Tumors in Rats Exposed Prepubertally to Genistein

The emphasis of pointing out that these experimental animals receive BPA or genistein exposure only during the prepubertal period is to point out that these changes in susceptibility for mammary cancer are effected early in postnatal life and appear to be permanent manifestations.

We hypothesize that these hormonally-active chemicals elicit developmental alterations that result in the biochemical “blue print” being permanently altered and differentially expressed later in life even in the absence of the original effector.

These alterations are termed organizational or imprinting effects.

Is there a precedence for this in the human population?

Yes.

Shu et al. investigated soyfood intake during adolescence and subsequent risk of breast cancer among Chinese women and found that girls eating soy prior to age 15 had a lower incidence of breast cancer than those not on a soy diet. (Cancer Epidemiol Biomarkers Prev 2001, 10:483–488.)
Mammary Gland Proteomics

Representative 2-D gel protein profile with the protein spots marked as differentially regulated from mammary glands of rats exposed to genistein. The identities of these spots were identified by MALDI-TOF-TOF and/or LC-MS/MS. These proteins were subsequently confirmed by western blot analysis.
Bioinformatic analysis on protein expression studies suggested that BPA and genistein regulate cell proliferation and apoptosis in the mammary gland.

Ki-67 staining for cell proliferation

Apop-Tag assay for apoptosis
Cell proliferation in mammary glands of 50-day-old rats exposed prepubertally to BPA and/or genistein, and SO (Controls) from day 2 until day 20 postpartum. Ki-67 expression was measured as an indicator of cell proliferation. Values represent mean ± SE; n = 6. a,b,c $p \leq 0.01$ compared with control. d $p \leq 0.001$ compared with BPA.

Jun Wang
Rate of cell apoptosis in mammary glands of 50-day-old rats exposed prepubertally to BPA and/or genistein, and SO (Controls) from day 2 until day 20 postpartum. ApopTag staining and morphological analyses were used as indicator of cell apoptosis. Values represent mean ± SE; $n = 6$. $^{a,b,c} p \leq 0.01$ compared with control. $^{d} p \leq 0.001$ compared with BPA.

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Protein expressions of SRC-1, SRC-2 and SRC-3 from mammary gland extracts of 50 day old rats exposed prepubertally to BPA or/and genistein, and SO (control). Densitometric values of Western blots were reported as a percentage of the controls ± SEM: a,b p ≤ 0.05 compared with control; c p ≤ 0.0001 compared with BPA. d p ≤ 0.05 compared with genistein.

**SRC:** Steroid Receptor Co-regulators (positive regulators of estrogen and progesterone receptor action) are associated with cell proliferation.
Prepubertal BPA or Genistein Effects in Mammary Glands of 50 Day Old Rats

Venn diagram depicting rate of cell proliferation and apoptosis, and differential regulation of associated proteins in mammary glands of 50 day old rats exposed prepubertally to BPA or genistein compared to controls.

This data supports our hypothesis that susceptibility for chemically induced mammary cancer can be predicted by the rate of cell proliferation and cell death.

Proteomic Biomarker Analysis in Blood of Preadolescent Girls Exposed to Bisphenol A and Genistein

Experimental Design

Urine → Measured (CDC): (phytoestrogens, phthalates, phenols)

Blood Serum

from Frank Biro, Susan Pinney, & Colleagues @ Cincinnati Children’s Hospital)

CCH to UAB

Proteomics

LC-MS/MS analysis (Jim Mobley et. al, UAB)

Data Analysis (Dong-quan Chen & Angela Betancourt, UAB)

Protein Biomarkers
Protein identification and quantification in serum from girls with high and low levels (control group) of endocrine active chemicals (Bisphenol A and genistein) in the urine. Human serum was immunodepleted of the 7 most abundant proteins and labeled with TMTs. Proteins were analyzed by on-line automated nano-LC-ESI-MS (SCX/ RP) MuDPIT (PQD-LTQXL ThermoFinnigan). Data were searched using SEQUEST, and analyzed using BioInQuires ProteoIQ software package.
Results

Summary of Proteins Identified to be Differentially Expressed in Serum of Pubertal Girls with High Urine Concentrations of BPA and Genistein

<table>
<thead>
<tr>
<th>Chemical</th>
<th>No. of serum proteins identified</th>
<th>No. of serum protein differentially expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPA</td>
<td>1992</td>
<td>51(2.6%)</td>
</tr>
<tr>
<td>Genistein</td>
<td>1364</td>
<td>34 (2.5%)</td>
</tr>
</tbody>
</table>
Bar graph representation of proteins classified by biological function. Analysis was carried out by PANTHER on differentially regulated proteins identified via TMT-MS from blood of prepubertal girls with high urine concentrations of BPA and genistein.
Differentially Regulated Cancer Associated Proteins Identified by TMT-MS in Blood of Girls with High Urine Concentrations of BPA

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein Name</th>
<th>Group Probability</th>
<th>No. Unique Peptides</th>
<th>Fold Change (Rx/C)</th>
<th>SAM a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q01484</td>
<td>ankyrin-2</td>
<td>0.99</td>
<td>4</td>
<td>+2.21</td>
<td>1.69</td>
</tr>
<tr>
<td>P46013</td>
<td>antigen Ki-67</td>
<td>0.95</td>
<td>4</td>
<td>+3.65</td>
<td>1.41</td>
</tr>
<tr>
<td>P45985</td>
<td>mitogen-activated kinase kinase 4 (MKK4)</td>
<td>0.95</td>
<td>2</td>
<td>+3.08</td>
<td>1.12</td>
</tr>
<tr>
<td>Q9Y4G6</td>
<td>talin-2</td>
<td>0.90</td>
<td>4</td>
<td>+2.54</td>
<td>1.43</td>
</tr>
<tr>
<td>Q9UL62</td>
<td>transient receptor potential channel 5 (TRPC5)</td>
<td>0.90</td>
<td>4</td>
<td>+2.40</td>
<td>1.23</td>
</tr>
<tr>
<td>Q96QB1</td>
<td>deleted in liver cancer 1 (DLC1)</td>
<td>0.83</td>
<td>3</td>
<td>-2.95</td>
<td>1.24</td>
</tr>
<tr>
<td>Q9UBC3</td>
<td>DNA (cytosine-5)-methyltransferase 3B (DNMT3B)</td>
<td>0.93</td>
<td>2</td>
<td>-2.51</td>
<td>1.77</td>
</tr>
<tr>
<td>Q6NUQ1</td>
<td>RAD50-interacting 1</td>
<td>0.92</td>
<td>2</td>
<td>-2.68</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Proteins identified using the Human Genome DB ID. Positive and negative fold change in protein expression indicate up- and down-regulation of protein expression relative to control, respectively. aSignificance analysis of microarray (SAM).
Differentially Regulated Cancer Associated Proteins Identified by TM-MS in Blood of Girls with High Urine Concentrations of Genistein

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein Name</th>
<th>Group Probability</th>
<th>No. Unique Peptides</th>
<th>Fold Change (Rx/C)</th>
<th>SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>P42892</td>
<td>endothelin-converting enzyme (ECE-1) 1</td>
<td>1.00</td>
<td>4</td>
<td>-2.12</td>
<td>1.72</td>
</tr>
<tr>
<td>B4DUI3</td>
<td>eukaryotic translation initiation factor 3 subunit J (EIF3) has been found elevated in human breast, cervical, esophageal, and lung cancers.</td>
<td>0.82</td>
<td>2</td>
<td>-2.30</td>
<td>1.63</td>
</tr>
<tr>
<td>Q9UMY1</td>
<td>nucleolar 7 is reported to be a candidate tumor suppressor gene in cervical cancer</td>
<td>0.82</td>
<td>2</td>
<td>+2.10</td>
<td>1.02</td>
</tr>
<tr>
<td>Q9NOX1</td>
<td>PR domain zinc finger 5 (PRDM5) has growth suppressive activities and is silenced in breast, ovarian, liver, lung, colon, and other cancers.</td>
<td>0.97</td>
<td>5</td>
<td>+2.30</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Proteins identified using the Human Genome DB ID. Positive and negative fold change in protein expression indicate up- and down-regulation of protein expression relative to control, respectively. "Significance analysis of microarray (SAM)."
Summary

• Our data demonstrates that prepubertal only exposure to BPA predisposes for chemically-induced mammary cancer in rats.

• On the other hand, prepubertal exposure to genistein via the diet suppresses chemically-induced mammary cancer in rats.

• Through the use of 2-D gels and MS, we demonstrate the separation and quantification of proteins in mammary glands of rats exposed to two hormonally-active chemicals.

• Based on differential protein expression occurring 30 days after last exposure to genistein and BPA we hypothesize that prepubertal exposure resulted in organizational effects, perhaps epigenetic alterations.

• Epigenetic studies demonstrate that prepubertal BPA and genistein exposures differentially alter methylation patterns of promoter regions of genes involved in regulating cell proliferation, apoptosis and carcinogenesis in the mammary glands of adult rats.
Summary continuation

- Using blood of girls with high urine concentrations of Bisphenol A and genistein, we show differentially expressed protein profiles. For this we used TMT-MS proteomic technology.
- Using western blot analysis on mammary glands of rats we have also demonstrated cross-species and tissue validation of differentially regulated proteins from the girls’ blood.
- These data suggest that proteomics is the future of cancer biomarker discovery because proteins (enzymes, growth factors, etc.) are the actual players in cause and prevention of cancer.
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