EPIGENETIC TRAITS AS BIOMARKERS OF CARCINOGENESIS

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Carcinogenicity assessment: current state and challenges

Epigenetic alterations during mouse liver carcinogenesis induced by N,N-diethylnitrosamine (DEN), carbon tetrachloride (CCl₄) or DEN+ CCl₄

Population-based epigenetic approach for identification of susceptible subpopulation to disease and exposure to toxicants

Epigenetic biomarkers for carcinogenicity assessment: fiction or reality?
Carcinogenicity testing – modern era challenges

- ≥ 65,000 chemicals have been manufactured for commercial use in industrialized countries
  *Eaton and Gallagher, 1997*

- ≥ 700 chemicals tested by NTP and RF in two-year bioassays

- ~ 31% of marketed drugs have not been tested according to present carcinogenicity testing guidelines
  *Brambilla and Martelli, 2009*

- 50% are carcinogens
- 50% are non-genotoxic carcinogens
- 50% are genotoxic carcinogens

- Most compounds presented to regulatory agencies today are non-genotoxic carcinogens

- No reliable rapid test for non-genotoxic chemicals

- Liver is a sensitive organ to non-genotoxic chemicals
Systems biology approach to carcinogenicity testing and cancer risk assessment

IS IT TIME FOR INCORPORATING EPIGENETIC EVALUATION IN CARCINOGENICITY TESTING AND CANCER RISK ASSESSMENT?

Zhang L. et al., Chemico-Biological Interactions, 2010
Carcinogen-DNA adducts as markers of exposure and cancer risk

EXPOSURE

REACTIVE METABOLITES

CARCINOGEN-DNA ADDUCTS

Environmental

Normal cells

Initiated cells

Endogenous

Benzo[a]pyrene

Benzo[a]pyrene-diol-epoxide (BPDE)

BPDE-DNA ADDUCTS

ACQUISITION OF ADDITIONAL RANDOM MUTATIONS

Clonal selection and expression of initiated cells

Mutator phenotype cells

Cancer cells

Hepatic tamoxifen–DNA adduct levels in F344 rats fed 420 ppm tamoxifen-containing diet for 6, 12, 18, or 24 weeks

<table>
<thead>
<tr>
<th>Weeks of feeding</th>
<th>dG-TAM</th>
<th>dG-DesMeTAM</th>
<th>Total TAM–DNA adducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>265 ± 13</td>
<td>318 ± 16</td>
<td>583 ± 21</td>
</tr>
<tr>
<td>12</td>
<td>493 ± 28</td>
<td>769 ± 43</td>
<td>1262 ± 66</td>
</tr>
<tr>
<td>18</td>
<td>631 ± 22</td>
<td>1020 ± 36</td>
<td>1651 ± 57</td>
</tr>
<tr>
<td>24</td>
<td>639 ± 56</td>
<td>1073 ± 87</td>
<td>1712 ± 143</td>
</tr>
</tbody>
</table>

The tamoxifen–DNA adduct levels are expressed as adducts/10^8 nt (n=5, mean ± SEM)
Possible mechanisms of chemical carcinogenesis

Miller, Cancer Res., 1970
Epigenetic alterations during mouse liver carcinogenesis induced by N,N-diethylnitrosamine (DEN), carbon tetrachloride (CCl₄) or DEN+ CCl₄
Liver tumor incidence induced by DEN, CCl\textsubscript{4} or DEN+CCl\textsubscript{4}
Liver fibrosis markers in mice treated with DEN, CCl₄ or DEN+CCl₄

PBS
DEN

Olive Oil

CCl₄

αSma-positive area (%)

Fold Change

Timp1 mRNA

Fold Change

17 wks
22 wks

PBS           DEN           CCl₄         DEN+CCl₄

*
Effects of DEN, CCl₄ or DEN+ CCl₄ on liver DNA methylation
Effects of DEN, CCl₄ or DEN+ CCl₄ on liver histone modifications

Global histone methylation

A

H3K9me3

17 wks

22 wks

Histone H3

Percent Control

PBS DEN CCl4 DEN+CCl4

B

H3K27me3

17 wks

22 wks

Histone H3

Percent Control

PBS DEN CCl4 DEN+CCl4

C

H4K20me3

17 wks

22 wks

Histone H4

Percent Control

PBS DEN CCl4 DEN+CCl4

Repetitive element histone methylation

LINE1

Fold Change

SINEB2

Fold Change

Major Satellites

Minor Satellites

Expression of repetitive elements

LINE1

Fold Change

SINEB2

Fold Change

Major Satellites

Minor Satellites
Promoter methylation in human hepatocellular carcinoma

Study design
- DNA Isolation
- Bisulfite Conversion
- Methylation-Specific PCR
- Survival analysis, correlations

Tumorous samples (n=43)
- p16**: 34/43 (79%)
- SOCS-1: 39/43 (91%)
- RASSF1A*: 43/43 (100%)
- APC**: 40/43 (93%)
- GSTP1**: 23/43 (53%)
- RIZ1**: 33/42 (79%)
- MGMT*: 0/43 (0%)

Non-Tumorous samples (n=45)
- p16**: 7/45 (16%)
- SOCS-1: 41/45 (91%)
- RASSF1A*: 31/45 (69%)
- APC**: 9/45 (20%)
- GSTP1**: 2/45 (4%)
- RIZ1**: 4/45 (9%)
- MGMT*: 7/45 (16%)

Hypomethylation of LINE-1 elements in tumorous but non-tumorous tissue samples
- Percent LINE-1 unmethylation (SD)
  - Tumorous samples (n=43): 64.4 (20.8)
  - Non-Tumorous samples (n=45): 42.7 (21.6)

Frequency of promoter methylation of tumor suppressor genes tumorous and non-tumorous liver DNA

Multiplicity of methylated genes in tumorous, non-tumorous and control liver DNA
- Tumorous (n=43)
- Non-Tumorous (n=45)
- Control (n=10)
Kaplan-Meier survival curves based on promoter hypermethylation of *Riz1* and *p16^{INK4A}* tumor suppressor genes.
Population-based epigenetic approach for identification of susceptible subpopulation to disease and exposure to toxicants
Nonalcoholic fatty liver disease (NAFLD) - the most frequent chronic liver disease in developed countries

NAFLD is a hepatic manifestation of the metabolic syndrome

NAFLD is associated with insulin resistance, obesity, and dyslipidemia

The majority of hepatocellular carcinomas in the United States are attributed to NAFLD

Nonalcoholic steatohepatitis (NASH) is the advanced stage of NAFLD

NASH pathophysiological features:
- Intrahepatic triglyceride accumulation
- Oxidative stress
- Liver injury
- Liver inflammation
- Apoptosis

The relation between NAFLD, NASH and HCC

30% of population has NAFLD (90 million)
10% of NAFLD develop NASH (9 million)
25% of NASH develop cirrhosis (2.25 million)
10-25% HCC

So, 200,000-500,000 potentially at risk in US

Siegel A.B. et al., Cancer, 2009
Methyl donor-deficient model of endogenous rodent liver

- Chronic deficiency in the methyl donors methionine, choline, folic acid and vitamin $B_{12}$
- No exogenous carcinogen added
- No genetic manipulation
- Hepatocellular carcinoma in 14-16 months in male rats and certain mouse strains
- Sequence of pathological changes similar to the development of hepatocellular carcinoma (HCC) in humans

[Diagram showing the sequence of changes from normal liver to HCC]

Normal tissue | 36 weeks, GST$\pi$-foci | Liver tumor
F344 SINGLE DEFINED GENOME

Genetically diverse human population

Susceptible to disease or exposure individuals
NOD/LtJ
A polygenic model for type 1 (non-obese) diabetes
Displays extremely long life-span

129S1/SvImJ
Used to make embryonic stem cell lines

C57BL/6J
Used for knockout models

CAST/EiJ
Resistant to cancer

A/J
An asthma model

PWK/PhJ
Susceptibility to type I diabetes and various behavioral traits

WSB/EiJ
Gathered from: http://www.sanger.ac.uk/mousegenomes
Total liver pathology scores of hepatic lesions in mice fed a choline- and folate-deficient diet

**Liver lesions**
- Steatosis
- Necrosis, hepatocyte
- Inflammation
- Oval cell hyperplasia
- Karyomegaly

All animals are equal, but some animals are more equal than others

GEORGE ORWELL, Animal Farm (1945)
Association between hepatic DNA methylome and severity of NAFLD-associated liver injury
Association between hepatic DNA methylome and severity of NAFLD-associated liver injury

C

Number of differentially methylated CpG islands in DNA from the livers of A/J, 129S1/SvImJ and WSB/EiJ mice

<table>
<thead>
<tr>
<th>Location</th>
<th>Methylation</th>
<th>A/J</th>
<th>129S1/SvImJ</th>
<th>WSB/EiJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter</td>
<td>HyperMe</td>
<td>115</td>
<td>56</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>HypoMe</td>
<td>150</td>
<td>209</td>
<td>230</td>
</tr>
<tr>
<td>Inside</td>
<td>HyperMe</td>
<td>286</td>
<td>150</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>HypoMe</td>
<td>203</td>
<td>339</td>
<td>388</td>
</tr>
<tr>
<td>Downstream</td>
<td>HyperMe</td>
<td>19</td>
<td>11</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>HypoMe</td>
<td>10</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>Divergent promoter</td>
<td>HyperMe</td>
<td>16</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>HypoMe</td>
<td>24</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>HyperMe</td>
<td>436</td>
<td>223</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>HypoMe</td>
<td>387</td>
<td>600</td>
<td>680</td>
</tr>
</tbody>
</table>
DNA methylation changes in the livers of control mice and mice fed a choline- and folate-deficient diet
DNA methylation changes in the livers of control mice and mice fed a choline- and folate-deficient diet: hunt for biomarkers

- **Common genes**: 567
- **Common genes correlating with the extent of liver injury**: 234
- **Common genes correlating with liver injury and gene expression**: 38
MicroRNA expression changes in the livers of control mice and mice fed a choline- and folate-deficient diet
MicroRNA levels in plasma of control mice and mice fed a choline- and folate-deficient diet
Correlation plots of total liver pathology scores and induction of miRNAs in the livers of mice fed a choline- and folate-deficient diet.
Correlation plots of total liver pathology scores and miRNA levels in plasma of mice fed a choline- and folate-deficient diet

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Correlation Coefficient</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-122</td>
<td>r = 0.573</td>
<td>p = 3.38 x 10^{-4}</td>
</tr>
<tr>
<td>miR-34a</td>
<td>r = 0.861</td>
<td>p = 8.68 x 10^{-11}</td>
</tr>
<tr>
<td>miR-200b</td>
<td>r = 0.394</td>
<td>p = 2.10 x 10^{-2}</td>
</tr>
<tr>
<td>miR-192</td>
<td>r = 0.628</td>
<td>p = 6.97 x 10^{-6}</td>
</tr>
<tr>
<td>miR-221</td>
<td>r = 0.604</td>
<td>p = 1.52 x 10^{-4}</td>
</tr>
<tr>
<td>miR-181a</td>
<td>r = 0.569</td>
<td>p = 4.48 x 10^{-4}</td>
</tr>
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Epigenetic biomarkers for carcinogenicity assessment: fiction or reality?
Biomarker requirements for carcinogenicity assessment

- Early appearance
- Stability
- Sensitivity
- Target tissue-specificity
- Applicability to both genotoxic and non-genotoxic carcinogenic agents
- Relatively low cost of the assays needed to detect these changes
- Mechanistic value

- Identify individuals with different susceptibility to disease, exposure, and drug response
Genotoxic and epigenetic changes in the livers of C57BL/6J mice exposed to 1,3-butadiene

**THB-Gua-DNA adducts**

Liver

<table>
<thead>
<tr>
<th></th>
<th>THB-Gua/10^8 nucleotides</th>
<th>% from control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td><img src="Liver_THB-Gua.png" alt="Graph" /></td>
<td>*</td>
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</tbody>
</table>

Kidney

<table>
<thead>
<tr>
<th></th>
<th>THB-Gua/10^8 nucleotides</th>
<th>% from control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kidney</strong></td>
<td><img src="Kidney_THB-Gua.png" alt="Graph" /></td>
<td>*</td>
</tr>
</tbody>
</table>

**Methylation of DNA repetitive elements**

LINE 1

<table>
<thead>
<tr>
<th></th>
<th>% from control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LINE 1</strong></td>
<td><img src="LINE_1.png" alt="Graph" /></td>
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</table>

SINE B1

<table>
<thead>
<tr>
<th></th>
<th>% from control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SINE B1</strong></td>
<td><img src="SINE_B1.png" alt="Graph" /></td>
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</tbody>
</table>

SINE B2

<table>
<thead>
<tr>
<th></th>
<th>% from control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SINE B2</strong></td>
<td><img src="SINE_B2.png" alt="Graph" /></td>
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</tbody>
</table>
Genotoxic and epigenetic changes in the livers of C57BL/6J and A/J mice exposed to 1,3-butadiene

**THB-Gua-DNA adducts**

<table>
<thead>
<tr>
<th>C57BL/6J</th>
<th>A/J</th>
</tr>
</thead>
<tbody>
<tr>
<td>THB-Gua/10^6 nucleotides</td>
<td><img src="image1" alt="Graph" /></td>
</tr>
</tbody>
</table>

**Global DNA Methylation**

<table>
<thead>
<tr>
<th>C57BL/6J</th>
<th>A/J</th>
</tr>
</thead>
<tbody>
<tr>
<td>% from control</td>
<td><img src="image3" alt="Graph" /></td>
</tr>
</tbody>
</table>

**LINE 1 Methylation**

<table>
<thead>
<tr>
<th>C57BL/6J</th>
<th>A/J</th>
</tr>
</thead>
<tbody>
<tr>
<td>% from control</td>
<td><img src="image5" alt="Graph" /></td>
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</tbody>
</table>

**H4K20me3**

<table>
<thead>
<tr>
<th>C57BL/6J</th>
<th>A/J</th>
</tr>
</thead>
<tbody>
<tr>
<td>% from control</td>
<td><img src="image7" alt="Graph" /></td>
</tr>
</tbody>
</table>
### Comparison between genetic and epigenetic aberrations in cancer

<table>
<thead>
<tr>
<th></th>
<th>Mutation</th>
<th>DNA methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of alterations per cancer cell</strong></td>
<td>~80, &lt;15 driver mutations</td>
<td>Several hundred to 1000</td>
</tr>
<tr>
<td><strong>Frequency of alterations of a specific gene in non-cancerous tissues</strong></td>
<td>$10^{-5}$/cell, up to $10^{-3}$/cell</td>
<td>From 0.1% up to 10% of cells</td>
</tr>
<tr>
<td><strong>Inducers</strong></td>
<td>Mutagenic chemicals, radiation, UV, oxygen radicals</td>
<td>Chronic inflammation, aging, mutagenic and <strong>non-mutagenic</strong> chemicals, radiation, UV, oxygen radicals</td>
</tr>
<tr>
<td><strong>Target gene</strong></td>
<td>Random</td>
<td>Specific</td>
</tr>
<tr>
<td><strong>Reversibility</strong></td>
<td>Irreversible</td>
<td>Reversible</td>
</tr>
</tbody>
</table>

Ushijima T, Asada K. Cancer Sci., 2010
Biomarker requirements for carcinogenicity assessment: *epigenetic vs genetic*

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Epigenetic</th>
<th>Genetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early appearance</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stability</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A greater number of detectable epigenetic changes as compared to the genetic alterations after exposure</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Target tissue-specificity</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Applicability to both genotoxic and non-genotoxic carcinogenic agents</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Relatively low cost of the assays needed to detect these changes</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mechanistic value</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Identify individuals with different susceptibility to disease, exposure, and drug response</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Summary

Epigenetic alterations are a major component and driving force in liver carcinogenesis. Our data indicate clearly that epigenetic alterations account for the formation and progression of preneoplastic lesions. Additionally, these results demonstrate that epigenetic alterations are early indicators of carcinogenic exposure and may be used for a carcinogenicity assessment and cancer prevention.
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