Cancer Driver Mutations in Experimental Rodents and Prediction of Human Cancer

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Long-standing unmet need for alternative approaches to the two-year rodent tumor bioassay

Current objectives:
• To develop panels of cancer driver mutational targets to serve as reporters of carcinogenic effects

Application:
• Incorporate analyses of cancer driver mutations into 28-day to 6-month repeat-dose rodent studies

Long-term goal:
• Predict rodent life-time carcinogenicity from shorter-term exposures
What defines cancer driver mutations (CDMs)

- Mutations causally implicated in oncogenesis
- Confer a growth advantage on the cells carrying them
- Positively-selected in the microenvironment of the tissue in which the cancer arises
- **Driver mutations tend to cause clonal expansions**

Strength of CDMs as biomarkers

- Clonal expansion of CDMs, which is integral to tumorigenesis, will make CDMs sensitive biomarkers of effect
- Clonal expansion of CDMs is a tumor-proximate biomarker of effect
- Potential reporters for genotoxic and non-genotoxic carcinogens

Human Relevance

- CDMs are being used in oncology, consequently prevalence and functional impacts of CDMs are being investigated intensively
- NGS analyses of pre-existing clones carrying CDMs in human samples are being reported
- DNA-based CDM measurements can be performed on any tissue of any species
- Identify CDMs with similar tissue-specific selective advantage in human and rodents
Allele-specific competitive blocker PCR (ACB-PCR) measurement of $p53$ R270C induction in mouse skin by simulated solar light (SSL)

Wild-type allele

TCGT
×GTA
BP

UP
AGCA

Little or no amplification of wild-type allele

Mutant allele

TTGT
↓ATA
MSP

UP
AACA

Selective amplification of mutant allele

$r^2 = 0.995$
Analysis of $p53$ mutations in response to dose of SSL in SKH-1 mouse skin

The increase in $p53$ mutant fraction (MF) was described by the linear function: 
\[ \log_{10} MF = \alpha + 0.0016 \cdot d, \]
where $\alpha$ is the spontaneous $\log_{10}$ MF at a given timepoint and $d$ is the dose of SSL in mJ·CIE/cm$^2$. 

![Graph showing the relationship between cumulative dose and $p53$ mutant fraction for different timepoints (4, 16, and 28 weeks).]
<table>
<thead>
<tr>
<th>Carcinogen</th>
<th>Strain/species, sex, age and number of treated rodents/group</th>
<th>Study design, route of exposure and dosing</th>
<th>Induced CDM and organ analyzed</th>
<th>Expression period or duration for significant induction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Aminobiphenyl</td>
<td>Mouse/C57Bl/6N, male, PND 8, n=12 Mouse/B6C3F1, Male, PND 8, n=12</td>
<td>Acute i.p dosing, 1/3 i.p. on PND8 and 2/3 on PND15, 0 or 0.3 µmole total dose</td>
<td>Hras Q61K in liver</td>
<td>Expression time: 32 weeks</td>
<td>Parsons et al. (2005) Molecular Carcinogenesis 42: 193-201</td>
</tr>
<tr>
<td>N-hydroxy-2-acetylaminofluorene</td>
<td>Rat/Big Blue®, male, 6 weeks old, n=5 (control) n=4 (treated)</td>
<td>Sub-chronic, repeat dose, 4 i.p. doses of 0 or 25 mg/kg b.w. at 4-day intervals</td>
<td>Kras G12V Kras G12D in liver</td>
<td>Expression time: 10 weeks from first dose (8 from last dose)</td>
<td>McKinzie et al. (2006) Mutagenesis 21: 391-397</td>
</tr>
<tr>
<td>Simulated solar light</td>
<td>Mouse/SKH-1 hairless, female, 8 weeks old, n=3 (4 and 16 week) n=4 (28 week)</td>
<td>Sub-chronic, repeat dose, 5 days per week at 0, 6.85, 13.70 or 20.55 mJ.CIE/cm²</td>
<td>Tp53 R270C in skin</td>
<td>Duration of exposure: 4, 16 or 28 weeks</td>
<td>Verkler et al. (2008) Molecular Carcinogenesis 47: 599-607</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>Mouse/A/J, male, 7-9 weeks old, n=10</td>
<td>Acute i.p. dosing, 0, 0.05, 0.5, or 50.0 mg/kg bw</td>
<td>Kras G12D Kras G12C in lung</td>
<td>Expression time: 4 weeks</td>
<td>McKinzie et al. (2010) Environ. Mol. Mutagen. 51: 145-155</td>
</tr>
<tr>
<td>Azoxymethane</td>
<td>Rats/F344, male, 7 weeks old, n=6</td>
<td>Acute s.c. dosing 0 or 30 mg/kg bw, ½ at week 7, ½ at week 8</td>
<td>Kras G12V Kras G12D in colon</td>
<td>Expression times: 1, 8, 24 or 32 weeks</td>
<td>McKinzie et al. (2011) Environ. Mol. Mutagen. 52: 409-418</td>
</tr>
<tr>
<td>Aristolochic acid</td>
<td>Rat/Big Blue®, male, 6 weeks old, n=6</td>
<td>Sub-chronic, repeat dose, 5 days per week at 0, 0.1, 1.0, and 10.0 mg/kg bw by gavage</td>
<td>Kras G12D in kidney Hras Q61L in liver and kidney</td>
<td>Duration of exposure: 12 weeks</td>
<td>Wang et al. (2011) Mutagenesis 26: 619-628</td>
</tr>
<tr>
<td>Aristolochic acid</td>
<td>Mouse/Hupki, female, 2-4 months old, n=3 (3 or 12 days) n=5 (21 days)</td>
<td>Acute/sub-chronic, repeat dose, daily at 0 or 5 mg/kg bw</td>
<td>Hras Q61L in kidney and forestomach</td>
<td>Duration of exposure: 3 weeks</td>
<td>Wang et al. (2012) Environ. Mol. Mutagen. 53: 495-504</td>
</tr>
<tr>
<td>Ethylene oxide</td>
<td>Mouse/Big Blue®, male, 8 weeks old, n=10</td>
<td>Sub-chronic, repeat dose, 6 hours per day, 5 days per week at 0, 10, 50, 100 and 200 ppm by inhalation</td>
<td>Kras G12V Kras G12D in lung</td>
<td>Duration of exposure: 4 weeks</td>
<td>Parsons et al. (2013) Toxicological Sciences 136: 26-38</td>
</tr>
</tbody>
</table>
Relationships between cancer driver MFs and tumor responses

Study 1
4-aminobiphenyl
8 month expression time vs tumors @ 1 yr newborn mouse assay design: 0.3 umol 4-ABP- or DMSO-treated B6C3F1 and DMSO-treated C57Bl/6 mice

\[
\text{Log}_{10} \text{Geomean MF}
\]

\[
\begin{align*}
& r^2 = 0.8121 \\
& P = 0.2855
\end{align*}
\]

\[
\text{Percent Mice with Liver Tumors}
\]

\[
\text{Hras Q61K Geomean MF}
\]

Study 2
Simulated solar light
4 month repeat dose vs tumors @ 1 yr 0, 6.85 and 13.70 mJ-CLE/cm² SKH-1 mice

\[
\text{Log}_{10} \text{MF Standard Deviation}
\]

\[
\begin{align*}
& r^2 = 0.9729 \\
& P = 0.1054
\end{align*}
\]

\[
\text{Percent Mice with SCC in situ or SCC}
\]

\[
\text{Tp53 R270C Geomean MF}
\]

Study 3
Inhaled ethylene oxide
1 month repeat dose vs lung tumors @ 2 yrs 0, 50 or 100 ppm in B6C3F1 BigBlue mice

\[
\begin{align*}
& r^2 = 0.8296 \\
& P = 0.2709
\end{align*}
\]

\[
\text{Percent Mice with Lung Tumors}
\]

\[
\text{Kras G12D Geomean MF}
\]

\[
\begin{align*}
& r^2 = 0.9999 \\
& P = 0.0051
\end{align*}
\]

\[
\text{Percent Mice with Liver Tumors}
\]

\[
\text{Hras Q61K Log}_{10} \text{MF Std Dev}
\]

\[
\begin{align*}
& r^2 = 0.9002 \\
& P = 0.2046
\end{align*}
\]

\[
\text{Percent Mice with SCC in situ or SCC}
\]

\[
\text{Tp53 R270C Log}_{10} \text{MF Std Dev}
\]

\[
\begin{align*}
& r^2 = 0.8799 \\
& P = 0.2253
\end{align*}
\]

\[
\text{Percent Mice with Lung Tumors}
\]

\[
\text{Kras G12D Log}_{10} \text{MF Std Dev}
\]
CDMs are prevalent in normal tissues and interindividual variation identifies tissue-specific selective advantage
CDMs as biomarkers in a field cancerization view of carcinogenesis

CDMs disrupt normal tissue homeostasis and are substrates for chemical carcinogenesis

- Carcinogen-induced mutation
- Pre-existing mutation analyzed for clonal expansion

Selective Pressure

Clonal Cooperation

Clonal Recruitment

Early Clonal Expansion

Additional Clones/Mutations

Pseudomonoclonal tumors with minor subpopulations carrying CDMs
The path forward

Need datasets defining relationships between measurements from panels of hotspot CDMs and tumor responses for rodent and human
Developed a human amplicon panel and analysis by error-corrected NGS (EC-NGS)

- The panel contains 13 amplicons from 9 cancer driver genes - 973 bp with >30 mutational hotspots
- Amplicons conserved coding sequences (human, rat, and mouse)
- Analyzed 9 normal lung, 9 normal breast, 10 ductal carcinomas and 9 lung adenocarcinomas
- Unique molecular identifier sequences incorporated during a first-round PCR
- A single-strand consensus sequence approach used for error correction
Performance of a human EC-NGS method

- Sensitivity of $10^{-4}$
- Good correlation (Spearman $r = 0.9371$, $P < 0.0001$, $n = 28$) and no bias (Bland-Altman $\log_{10}$ bias = 0.00288) between ACB-PCR and EC-NGS MF measurements
- 50-100 mutations detected/sample, including expected hotspot CDMs and other COSMIC missense mutations
- Expected tissue-specific mutant enrichment for both tumor and normal
- Significant differences in mutational spectra and observed the expected predominant mutational specificities for breast and lung carcinomas
- Significant correlation between sum of MFs for each individual and tissue donor age
Conclusions

• Panels of hotspot CDMs appear to be promising biomarkers of carcinogenic effect and may be translatable between rodent and human

• Measurements of CDMs can simultaneously serve as reporters of carcinogenic effect and discern differences in mutation spectra

• Moving forward, need to define relationships between panels of CDM measurements and tumor responses for rodent and human

• Envision using panels of CDMs in a tissue-specific manner, during pre-clinical safety assessment of drugs or repeat-dose toxicity testing, focusing on tissues where pharmacological impacts are expected or toxicity is observed.

• Currently, developing rat and mouse amplicon panels analogous to human
  ▪ Validating rodent panels using rodents with known strain differences in spontaneous mammary and lung tumor susceptibility
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