ACB-PCR measurement of K-ras codon 12 mutant fractions in livers of Big Blue® rats treated with N-hydroxy-2-acetylaminofluorene

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K-ras codon 12 GGT→GAT and GGT→GTT mutations are the most frequently observed K-ras point mutations in human and rodent tumors and therefore are implicated in carcinogenesis for many tissues. Measurement of these mutations in rat models and human tissue could facilitate a more logical extrapolation of rodent tumorigenesis data to human disease. We have developed allele-specific competitive blocker PCR (ACB-PCR) assays for rat K-ras codon 12 GGT→GTT and GGT→GAT mutations that parallel the already published assays for human K-ras codon 12 mutations. Liver K-ras codon 12 mutant allele fractions were measured in vehicle-treated and N-hydroxy-2-acetylaminofluorene (N-OH-AAF)-treated Big Blue® rats. The average K-ras codon 12 GGT→GTT mutant fraction (MF) for four control rats was 50 × 10⁻⁶ (95% CI: 27 × 10⁻⁶, 95 × 10⁻⁶) and for four treated rats was 165 × 10⁻⁶ (95% CI: 87 × 10⁻⁶, 312 × 10⁻⁶), indicating a 3.3-fold increase with treatment (95% CI: 1.3–8.1). The average MF of K-ras codon 12 GGT→GAT for control rats was 1320 × 10⁻⁶ (95% CI: 498 × 10⁻⁶, 3500 × 10⁻⁶) and for treated rats was 8450 × 10⁻⁶ (95% CI: 3180 × 10⁻⁶, 22 400 × 10⁻⁶), indicating a 6.4-fold increase with treatment (95% CI: 1.6–25.4). These transgenic rats were the most frequently observed K-ras point mutations in many cancers, it is an important target to measure.

ACB-PCR method both K-ras codon 12 GGT→GTT and GGT→GAT MFs were significantly increased in treated rats versus control rats. This data raises the possibility that N-OH-AAF may not only induce mutations by a genotoxic mechanism, but also by amplification of both de novo and pre-existing K-ras mutation.

Introduction

Non-hereditary carcinogenesis is generally regarded as a combinatorial process with multiple discrete steps. This process is thought to be initiated by DNA damage upon exposure to a carcinogen, with misrepair of a fraction of the DNA lesions. These misrepaired lesions then become persistent mutations. Some of these mutations are in genes that result in altered cellular functions, producing phenotypic changes that are ultimately manifested as visible lesions.

Rodents are often used as animal models for human exposure, risk and disease. Some of the most useful animal models for studying mutagenesis are transgenic rats and mice engineered to carry the Escherichia coli lacI gene shuttle vector (1). These animals are used for analyzing in vivo somatic cell mutations in any tissue from which sufficient DNA can be recovered. Transgenic rats carrying the lacI shuttle vector (e.g. Big Blue® rats) can be used to determine the frequencies and types of persistent in vivo mutations caused by exposure to test compounds (2–5). However, mutations in the lacI gene do not confer selective growth advantage or other functional changes associated with carcinogenesis, so the relationship of these mutations to proto-oncogene or tumor suppressor gene mutations is not well-defined.

Many of the proto-oncogene and tumor suppressor genes involved in human diseases also are involved in disease processes in animal models, such as the K-ras gene. K-ras codon 12 mutations are frequently observed in both human and rat tumors, with the GGT→GTT and GGT→GAT mutations accounting for ~32% of observed K-ras codon 12 mutations in human lung, colon, pancreas and uterine tumors (6,7). Because of the predominance of the K-ras mutations in many cancers, it is an important target to measure.

We previously developed an allele-specific, DNA amplification method to measure human K-ras codon 12 point mutations. In this allele-specific competitive blocker PCR (ACB-PCR) assay, the mutant allele is preferentially amplified, while the amplification of the wild-type allele is minimized. Details of this assay are described elsewhere (8,9). Briefly, the assay includes three primers: an extendable mutant-specific primer (MSP), a non-extendable blocker primer (BP) and an extendable downstream primer. To reduce background amplification of the wild-type allele, the MSP is designed with a double mismatch at the 3'-end relative to the wild-type sequence. Although this creates a 3'-penultimate mismatch between the MSP and the mutant allele to be detected, amplification of the mutant allele is greatly favored over amplification of the doubly mismatched wild-type allele. Additionally, a non-extendable primer is added that preferentially anneals to the wild-type allele, blocking these sequences from being primed. The BP has a 3'-double mismatch relative to the mutant allele, so that the blocking of the mutant is minimized.

To date, the ACB-PCR method has been developed for the measurement of human K-ras codon 12 GGT→GAT and GGT→GTT mutations (9), mouse p53 codon 270 GGT→TGT mutation (10), and mouse H-ras codon 61 CAA→AAA mutation (11). In a study of H-ras mutation in mice treated with 4-aminobiphenyl (4-ABP), the utility of ACB-PCR was demonstrated by comparing the H-ras mutant fractions (MFs) induced in liver to the tumor burden of like-treated animals. The results indicated that livers of untreated B6C3F1 male mice had an average MF of 1 × 10⁻⁵ and livers of treated animals had an average MF of 4.49 × 10⁻⁵, a 34.5-fold increase.

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at 8 months after 4-ABP-treatment. A chemical-associated increase in H-ras codon 61 CAA—AAA MF was measurable by ACB-PCR and could be correlated with the 4-ABP-induced tumor response, confirming the usefulness of this assay for measuring cancer-related gene mutations.

Relatively little information is available regarding the role of K-ras codon 12 mutations in rat liver tumor development. A few studies have reported data on K-ras codon 12 mutations in rat liver tumors (12–14), with K-ras codon 12 mutations being found only in liver tumors induced by treatment with high doses of nitroglycerin (15). However, the most sensitive mutation detection method applied to the detection of K-ras liver mutation is single-strand conformation polymorphism (SSCP) analysis. The greatest sensitivity reported for SSCP is $3 \times 10^{-3}$ (16), whereas the sensitivity of ACB-PCR is $1 \times 10^{-7}$ (9–11). Prior to the current study there were no published data on K-ras mutations in liver and liver tumors of rats treated with N-OH-AAF, and no data describing K-ras mutations in normal-appearing liver tissue of rats treated with hepatic carcinogens.

The ACB-PCR assay for K-ras mutations was first developed for the measurement of human K-ras codon 12 GGT—GTT and GGT—GAT mutations. With the goal of using ACB-PCR data to inform species extrapolation in cancer risk assessment, we developed assays for quantifying rat K-ras codon 12 GGT—GTT and GGT—GAT mutations in order to compare the role of K-ras mutations in rat models to human disease. The utility of the developed ACB-PCR assays were then demonstrated in the measurement of mutations in rat liver genomic DNA isolated from a previous study of Big Blue® rats treated with N-hydroxy-2-acetylamino-fluorenone (N-OH-AAF) (17,18). This approach also provides a direct comparison between the mutational responses in a transgenic reporter locus and an endogenous cancer-associated gene.

### Materials and methods

#### Animal treatment

Liver tissue from a previous study (17,18) was used for the ACB-PCR assays. Male 6-week-old Big Blue® rats were purchased from Taconic Farms (Germantown, NY). We followed the recommendations set forth by our Institutional Animal Care and Use Committee for the handling, maintenance, treatment and termination of the animals. The animals were given four intraperitoneal (i.p.) doses of 25 mg K-ras intraperitoneal (i.p.) delivered in propane-1,2-diol at 4-day intervals (17). An age-matched control group was dosed with four i.p. injections at 4-day intervals with vehicle only (propane-1,2-diol). The i.p. injections were in a volume of 0.5 ml/kg body weight. The rats were sacrificed by CO2 exposure 10 weeks after the initial dose, at age 16 weeks. Livers were collected at necropsy, frozen on dry ice and stored at −80°C.

#### DNA isolation

Genomic DNA was isolated from rat liver tissue as previously described (19), digested overnight with the EcoRI restriction enzyme (New England Biolabs, Ipswich, MA), extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and the DNA precipitated in 100% ethanol. The DNA was resuspended in 0.5x Tris-EDTA buffer (5 mM Tris-HCl, 0.5 mM EDTA, pH 7.5) and the concentration measured by UV absorbance at 260 nm.

#### Preparation of K-ras codon 12 wild-type standard and K-ras PCR product from liver samples

DNA isolated from liver of a male 3-week-old F344 rat was used as template in a first-round PCR to produce the wild-type template for the ACB-PCR assay. Because the final sensitivity of the ACB-PCR assay can be no better than the error rate of the DNA polymerase used in the first-round PCR, the high-fidelity enzyme Pfu (Stratagene, La Jolla, CA) was used for amplification of standards and samples. A 304 bp fragment encompassing K-ras exon 1 was amplified in the first-round PCR. Each 100 μl reaction contained: 1 μg of template DNA, 1x Pfu buffer, 0.2 mM each dATP, dTTP, dGTP, dCTP, 1 μM KRR2 (downstream primer, 5’-CTGTACCGATGGTCTCCT-3’), 1 μM KRR5 (upstream primer, 5’-ATATTTATTTTTTATTATAAAGCCTG-3’), 10 μl Pfu Ultra HotStart (Stratagene). The primers were purchased from Sigma-Genosys (Woodlands, TX). The cycling conditions included a preincubation at 94°C for 2 min, followed by 37 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C. The same PCR conditions were used to amplify the K-ras sequence from the livers of control and treated animals.

#### Preparation of K-ras codon 12 mutant standards

The GAT and GTT mutant standards were generated by in vitro mutagenesis. The reaction conditions for the first-round PCR of the mutant standards (100 μl) were: 1 μg of template DNA, 1x Pfu buffer, 0.2 mM each dATP, dTTP, dGTP, dCTP, 1 μM KRR2 (downstream primer, 5’-CTGTACCGATGGTCTCCT-3’), 1 μM mutagenic primer, 10 μl Pfu Ultra HotStart (Stratagene). The cycling conditions were initiated with a pre-incubation at 94°C for 2 min, followed by 37 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C. The sequence of the GTT mutagenic primer was 5’-ATATTTATTTTTTATTATAAAGCCTGCTCCTAATAACCTGTGGTAGTTGGAGCT-3’.

#### First-round PCR product verification and isolation

The size of the first-round PCR products of all standards and samples were verified by electrophoresis in 1.2% agarose gels with ethidium bromide staining. Once verified, the first-round PCR products were purified by electrophoresis in 0.7% agarose gels. The 304 bp bands of DNA were excised from the agarose and the DNA isolated from the gel slices using a GENE CLEAN kit (Q-Biogene, Irvine, CA). The isolated DNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

#### ACB-PCR conditions

Using the mutant and wild-type DNA fragments produced in the first-round PCR, MF standards of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$ were prepared. The MF standards contained a final concentration of 0.5 x $10^{14}$ K-ras codon 12 copies per μl. For the ACB-PCR assay, 4 μl of each standard was used, resulting in a total of 2 x $10^{14}$ K-ras copies in each reaction, and the same number of molecules (produced in the first-round PCR) were analyzed for each unknown sample. Two controls were also included in each ACB-PCR assay: a no-mutant control consisting of 2 x $10^{14}$ copies of wild-type K-ras standard, and a no-DNA control consisting of 4 μl of water instead of template. The ACB-PCR reactions were set up as previously described including the same primers (9), but with the specific reaction and cycling conditions optimized for the rat K-ras codon 12 GGT—GTT and GAT mutagenic standards (Table I). The method requires three primers; a downstream primer, a 5’ fluoresein-labeled MSP, and a BP terminating in a 3’-dideoxynucleotide. All three primers were purchased from Sigma Genosys, with the MSPs and BPs ordered as purified by PAGE. The ACB-PCR were initiated using a hot-start procedure by the addition of AmpliTaq DNA Polymerase Stoffel fragment (Applied Biosystems, Foster City, CA) and PerfectMatch™ PCR enhancer (Stratagene).

#### Analysis of ACB-PCR products

Equal volumes of ACB-PCR products were analyzed by PAGE using 8% polyacrylamide/TAE gels run for 2.5 h at 200 V. In the initial assay for each mutant, the size of the ACB-PCR products were verified against a DNA molecular weight ladder and subsequent staining with VistraGreen® (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) (data not shown). Due to incorporation of the fluorescein-labeled MSP, the K-ras PCR product is fluorescent and the fluorescein-labeled DNA product band in each subsequent assay was detected using a FluorImage™ (GE Healthcare Bio-Sciences Corp.) and quantified using ImageQuant™ (GE Healthcare Bio-Sciences Corp) software. The fluorescence detected from the MF standards was quantified as number of pixels by the ImageQuant™ software and used to construct

### Table 1. ACB-PCR conditions for rat K-ras codon 12 GGT—GTT and GAT—GAT MF measurements

<table>
<thead>
<tr>
<th>Mutant</th>
<th>dNTP (μM)</th>
<th>MgCl₂ (mM)</th>
<th>MSP (nM)</th>
<th>DSP (nM)</th>
<th>BP (nM)</th>
<th>PM (μM/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGT</td>
<td>40</td>
<td>1.5</td>
<td>400</td>
<td>400</td>
<td>300</td>
<td>0.09</td>
</tr>
<tr>
<td>GAT</td>
<td>80</td>
<td>1.6</td>
<td>500</td>
<td>500</td>
<td>325</td>
<td>0.07</td>
</tr>
</tbody>
</table>

MSP, mutant specific primer; DSP, downstream primer; BP, blocker primer, PM, Perfect Match®.
A standard curve. Three replicate experiments were performed to quantify the \( K\text{-ras} \) codon 12 GGT to GTT and GGT to GAT MFs in each liver DNA sample.

**Statistical analysis**

A standard curve for each assay was constructed by taking the logarithm of the number of pixels reported for each standard sample and fitting the data with a linear regression. The number of pixels observed for each unknown sample was converted to log\(_{10}\) and the MF calculated using this calibration. Statistical analyses were performed using analysis of variance and the results reported with one-sided \( P \)-values. Data values of MFs \(< 1 \times 10^{-5}\) (below the limit of detection) were deleted from the analyses.

**Results**

Genomic DNA from \( N\)-OH-AAF-treated and control Big Blue\textsuperscript{®} rat livers was isolated and the \( K\text{-ras} \) codon 12 region amplified. The 304 bp fragment was quantified and used as template in the ACB-PCR analyses. A total of \( 2 \times 10^8 \) copies of the \( K\text{-ras} \) codon 12 fragment was analyzed for each sample, and the ACB-PCR assays were performed in triplicate for both the \( K\text{-ras} \) codon 12 GTT→GTT and GGT→GAT MF determinations.

A representative \( K\text{-ras} \) codon 12 GTT→GTT analysis is shown in Figure 1A. As expected, the fluorescently labeled ACB-PCR product is a 103 bp band. The pixel count from each standard band was transformed to its log\(_{10}\) base and plotted against the log\(_{10}\) base of its MF (Figure 1B). The equation describing the linear regression produced for these samples was then used to estimate the MFs of each sample. The estimations from three experiments, along with the mean and 95% confidence interval, are listed in Table II.

In both the GTT and GAT MF assays, one control animal (animal 1) in Tables II and III, had MFs significantly greater than the other control animals (GAT: \( P = 0.0069 \), GTT: \( P = 0.0009 \)) and greater than the treated animals (GAT: \( P = 0.0990 \), GTT: \( P = 0.0070 \)). The MF estimates for this animal are presumed to be valid, as they were repeated in the three assays. However, these data were excluded from the control group in subsequent analyses because the MF observed in this animal was significantly different from the other animals. If included in the control group, the average MFs observed in treated animals remained larger than that of control animals although these differences were no longer statistically significant.

Analyses of variance were conducted using the data in Tables II and III. As discussed above, all data from animal 1 was excluded. Additionally, two other assays were excluded because the estimated MF was below the limit of detection (\( 1.0 \times 10^{-5} \)) and are marked as excluded in Tables II and III. Analyses were on the logarithms of the MF because the variation among assays within animals is relatively constant on the logarithmic scale. The variation among animals within treatments was not significant relative to the variation between repeated assays of the same animals (GAT: \( P = 0.099 \), GTT: \( P = 0.682 \)). Most importantly, the mean MFs of the treated animals were significantly greater than the mean MF of the control animals relative to variation among animals within treatments for both mutations (GAT: \( P = 0.017 \), GTT: \( P = 0.018 \)). Sampling errors as defined by Poisson’s distribution,
in addition to pipetting errors and variability in the ACB-PCR amplification efficiency, contribute to the variability between the assays and has been discussed previously (7).

The average MF of K-ras codon 12 GGT ! GTT for the control rats was $2950 \times 10^{-6}$ (95% CI: $272 \times 10^{-6}$, $3330 \times 10^{-6}$) and for the treated rats was $165 \times 10^{-6}$ (95% CI: $87 \times 10^{-6}$, $312 \times 10^{-6}$), a 3.3-fold increase (95% CI: 1.3, 8.1). The average K-ras codon 12 GGT ! GAT MF for the four control rats was $1320 \times 10^{-6}$ (95% CI: $498 \times 10^{-6}$, $3500 \times 10^{-6}$) and for the four treated rats it was $8450 \times 10^{-6}$ (95% CI: $3180 \times 10^{-6}$, $22400 \times 10^{-6}$), a 6.4-fold increase (95% CI: 1.6, 25.4). Additionally, there is a striking difference in the levels of the two mutations, with control GAT MF being 26.4-fold greater than control GTT, and treated GAT MF 51.2-fold greater than the treated GTT MF.

### Discussion

The ACB-PCR method can quantify the fraction of K-ras mutant alleles in any tissue, which enables studies of K-ras MF measurements during the progression of carcinogenesis in rat models and compared to K-ras MF data from human tissue. The ACB-PCR method was developed for the measurement of rat K-ras codon 12 GGT ! GAT and GGT ! GAT MFs as was previously done for the human K-ras codon 12 mutations (9). The rat ACB-PCR conditions were similar, showing that this method is portable and is becoming easily adaptable in establishing assays with a sensitivity of $10^{-5}$ for new mutations. To validate the rat K-ras codon 12 GGT ! GTT and GGT ! GAT ACB-PCR assays, MFs were measured and compared in Big Blue® rat liver DNA samples of control and N-OH-AAF-treated rats (17). For the rat K-ras codon

### Table II. Measurement of K-ras codon 12 GGT—GTT MF in liver of Big Blue® rats

<table>
<thead>
<tr>
<th>Animal/treatment</th>
<th>Assay 1 (MF $\times 10^{-6}$)</th>
<th>Assay 2 (MF $\times 10^{-6}$)</th>
<th>Assay 3 (MF $\times 10^{-6}$)</th>
<th>Mean$^a$ (MF $\times 10^{-6}$)</th>
<th>Lower 95% bound (MF $\times 10^{-6}$)</th>
<th>Upper 95% bound (MF $\times 10^{-6}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/control</td>
<td>2950</td>
<td>522</td>
<td>3330</td>
<td>1720</td>
<td>436</td>
<td>6820</td>
</tr>
<tr>
<td>2/control</td>
<td>14</td>
<td>76</td>
<td>480</td>
<td>79</td>
<td>20</td>
<td>313</td>
</tr>
<tr>
<td>3/control</td>
<td>58</td>
<td>17</td>
<td>22</td>
<td>28</td>
<td>7</td>
<td>110</td>
</tr>
<tr>
<td>4/control</td>
<td>1.6$^c$</td>
<td>184</td>
<td>12</td>
<td>47</td>
<td>9</td>
<td>251</td>
</tr>
<tr>
<td>5/control</td>
<td>45</td>
<td>131</td>
<td>41</td>
<td>62</td>
<td>16</td>
<td>246</td>
</tr>
<tr>
<td>Mean$^{a,b}$/control</td>
<td>50</td>
<td>27</td>
<td>95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/treated</td>
<td>206</td>
<td>110</td>
<td>188</td>
<td>162</td>
<td>41</td>
<td>641</td>
</tr>
<tr>
<td>7/treated</td>
<td>109</td>
<td>41</td>
<td>524</td>
<td>133</td>
<td>34</td>
<td>524</td>
</tr>
<tr>
<td>8/treated</td>
<td>165</td>
<td>420</td>
<td>728</td>
<td>369</td>
<td>93</td>
<td>1460</td>
</tr>
<tr>
<td>9/treated</td>
<td>95</td>
<td>178</td>
<td>48</td>
<td>93</td>
<td>24</td>
<td>368</td>
</tr>
<tr>
<td>Mean/treated</td>
<td>165</td>
<td>87</td>
<td>312</td>
<td>87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fold change</td>
<td>3.3</td>
<td>1.3</td>
<td>8.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Geometric mean, i.e. mean of the logarithms converted back to original scale.

$^b$Excludes Animal 1.

$^c$Excluded from analyses.

Fig. 2. ACB-PCR assay of rat K-ras codon 12 GGT—GAT mutation. (A) PAGE of ACB-PCR MF standards and liver DNA samples. (B) Standard curve relating pixel intensity of the 103 bp ACB-PCR product to K-ras MF.
12 GGT→GTT mutation, the mean MF of control animal samples was \(5 \times 10^{-6}\) and for treated samples it was \(165 \times 10^{-6}\), a significant 3.3-fold increase (Table II). For the K-ras codon 12 GGT→GAT mutation, the mean MF of controls was \(1320 \times 10^{-6}\) and for treated it was \(8450 \times 10^{-6}\), a significant 6.4-fold increase (Table III).

The results from the liver K-ras mutation ACB-PCR measurements are herein compared with the results from the liver lacI studies previously reported (17,18). The ACB-PCR assay MF data can be logically compared to the lacI assay mutant frequency data because both assays report the fraction of a cell population containing a mutation. The lacI assay reports mutant frequency calculated from the ratio of mutant plaques to total plaques measured, and the ACB-PCR method reports the fraction of mutant alleles to the total population of alleles. However, some differences need to be pointed out before comparisons are made. First, the reported liver lacI mutation spectra were from livers of rats 6 weeks after initial dosing with four doses of 25 mg/kg body wt. N-OH-AAF (100 mg/kg body wt total). However, the ACB-PCR analyses were performed using liver samples of rats 10 weeks after initial dosing with four doses of 25 mg/kg body wt N-OH-AAF. This time point was chosen in order to validate the ACB-PCR assay with samples having the greatest likelihood of measurable K-ras MFs. It is not known if the mutant frequency of lacI is constant from 6 to 10 weeks after treatment; however, the hprt mutant frequency in spleen lymphocytes was not significantly different between the 6- and 10-week 100 mg N-OH-AAF dose samples (17), which suggests that the lacI gene mutation frequency may also be stable in the same time period.

The lacI mutant frequencies for the G→T and G→A mutants were calculated using the mutation spectra data and the overall mutant frequency data (Table IV). For control rats, the average lacI mutant frequency was \(25.7 \times 10^{-6}\), with G→T mutations accounting for 18% of these mutations. The G→T mutant frequency was estimated by multiplying the total mutant frequency by 0.18 resulting in a G→T mutant frequency of \(4.7 \times 10^{-6}\). The G→A mutations in control rats account for 41% of the control mutations, therefore the lacI G→A mutant frequency can be estimated as \(10.5 \times 10^{-6}\). In the treated group, the average mutant frequency was \(406.8 \times 10^{-6}\). The G→T mutations account for 34%, while G→A mutations account for 20% of the total mutations, giving estimated mutant frequencies of \(140 \times 10^{-6}\) and \(81 \times 10^{-6}\), respectively. The absolute mutant frequency per G:C basepair was then derived by accounting for the number of mutable G:C sites in the lacI transgene. Using data from the database described by Cariello et al. (20) and new G:C sites described in the N-OH-AAF treated animals (18), it is estimated that there are 152 mutable G:C sites. The calculated absolute mutant frequencies per basepair are reported in Table IV.

Comparison of the data in Tables II and III shows K-ras MFs are much greater than expected based upon the lacI assay results. The control K-ras codon 12 GGT→GTT and GGT→GAT measurements are \(50 \times 10^{-6}\) and \(1320 \times 10^{-6}\), 1700-fold and 19,000-fold greater than the lacI mutant frequency per mutable site, respectively. For the treated groups, the K-ras codon 12 GGT→GTT MF is \(165 \times 10^{-6}\) (180-fold greater than the lacI G→T MF per mutable site) and the GGT→GAT MF is \(8450 \times 10^{-6}\) (16,000-fold greater than lacI G→A mutant frequency per mutable site). This illustrates that a neutral exogenous gene and a potentially cancer-related endogenous gene are strikingly dissimilar and this is attributable to differences in de novo mutation, clonal growth.
of de novo mutation, and clonal expansion of pre-existing mutation.

Exposure to N-OH-AAF primarily induces G→T transversions in a variety of targets including bacterial and mammalian genes (21). Because of this mutational specificity, the induced K-ras GGT→GTT MF may provide an approximation of the chemically-induced de novo mutation and subsequent expansion of these de novo mutations at K-ras codon 12. When the GTT measurements are subtracted from the N-OH-AAF-induced K-ras GAT measurements, a MF of 4900×10^{−6}, or 98% of the K-ras GAT MF, remains unaccounted for. This suggests that the remaining GAT MF may represent expansion of pre-existing mutant cells, the presence of which is corroborated by the high level of K-ras GAT MF in control animals. This interpretation of the data supports the hypothesis that the increase of a specific mutation in a tumor after carcinogen exposure may be due in large part to expansion of pre-existing mutations (16,22).

In this study, the animal livers are still growing from adolescent to adult size. In this biological environment of growth during chemical treatment, the pre-existing mutant cells may gain a proliferative advantage until the liver is full size, thereby causing a greater K-ras MF in the treated animal livers versus the control animal livers. A more thorough investigation to discern clonal expansion from hot-spot de novo mutations requires a time-course study that includes an early time point after treatment and multiple small samples from each tissue to address the distribution of the mutant clones. Whether due to clonal expansion or hot-spot mutation, it is evident from the current study that treatment with N-OH-AAF increases the K-ras codon 12 GGT→GTT and GGT→GAT MFs in normal-appearing treated rat livers versus control rat livers. The K-ras mutant cells may have acquired one of the hallmarks of cancer, increased proliferation (23), which potentially constitutes a field from which tumorigenesis is more likely to proceed (7).

An anomaly is presented by the one control rat which has a K-ras codon 12 GGT→GAT MF of 49 800×10^{−6} and GGT→GAT MF of 879×10^{−6}, values greater than expected relative to the other control animals. The F344 rat rarely develops sporadic liver tumors, so this high MF level raises the question regarding the role of K-ras mutations in the carcinogenic process in rat liver. K-ras mutations may cause a cellular functional change of increased and continuous MAPK pathway activation, which may be a necessary, but not rate-limiting, part of the carcinogenic process (24). Tumor development and carcinogenesis is a complex process in which polyclonal interactions may be a major contributor to overall growth and selection advantage. The exponential increase in the number of aberrant cells during carcinogenesis may be due to the mutual advantage of different clones of cells growing together in close proximity (25–27).

The present study illustrates how the ACB-PCR assay for tumor-related mutations, such as K-ras mutations, has the potential to distinguish whether a chemical’s mode of action is due to de novo mutations (i.e. genotoxic) or due to expansion of pre-existing mutations (i.e. non-genotoxic). K-ras mutations are considered to be an integral part of tumorigenesis in a number of important cancers, and therefore is a relevant biological marker of both cause and effect of exposure to mutagenic compounds. The ACB-PCR method can quantify the fraction of K-ras mutant alleles in any tissue and provides both quantitation and a sensitivity of 1×10^{−5} that could be used for collecting data from low dose exposure experiments, as well as data for incorporation into a mathematical model of the carcinogenic process. Additionally, the ACB-PCR method has been developed for the human K-ras codon 12 GGT→GAT and GGT→GTT mutations so that parallel assays could be employed to compare the mutational load in rat and human tissues. ACB-PCR measurement of K-ras mutation in both species is an important initial step in making relevant extrapolations from a test animal model to human disease.

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References


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