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Authors
Besaratinia, Ahmad
Li, Haiqing
Yoon, Jae-In
et al.

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A high-throughput next-generation sequencing-based method for detecting the mutational fingerprint of carcinogens

Ahmad Besaratinia1*, Haiqing Li2, Jae-In Yoon1, Albert Zheng1, Hanlin Gao3 and Stella Tommasi1

1Department of Cancer Biology, 2Department of Molecular Medicine and 3DNA Sequencing/Solexa Core Facility, Beckman Research Institute of the City of Hope, 1500 East Duarte Road, Duarte, CA 91010, USA

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ABSTRACT

Many carcinogens leave a unique mutational fingerprint in the human genome. These mutational fingerprints manifest as specific types of mutations often clustering at certain genomic loci in tumor genomes from carcinogen-exposed individuals. To develop a high-throughput method for detecting the mutational fingerprint of carcinogens, we have devised a cost-, time- and labor-effective strategy, in which the widely used transgenic Big Blue®/C213 mouse mutation detection assay is made compatible with the Roche/454 Genome Sequencer FLX Titanium next-generation sequencing technology. As proof of principle, we have used this novel method to establish the mutational fingerprints of three prominent carcinogens with varying mutagenic potencies, including sunlight ultraviolet radiation, 4-aminobiphenyl and secondhand smoke that are known to be strong, moderate and weak mutagens, respectively. For verification purposes, we have compared the mutational fingerprints of these carcinogens obtained by our newly developed method with those obtained by parallel analyses using the conventional low-throughput approach, that is, standard mutation detection assay followed by direct DNA sequencing using a capillary DNA sequencer. We demonstrate that this high-throughput next-generation sequencing-based method is highly specific and sensitive to detect the mutational fingerprints of the tested carcinogens. The method is reproducible, and its accuracy is comparable with that of the currently available low-throughput method. In conclusion, this novel method has the potential to move the field of carcinogenesis forward by allowing high-throughput analysis of mutations induced by endogenous and/or exogenous genotoxic agents.

INTRODUCTION

The human cancer genome is shaped by assaults from endogenous and exogenous mutagens (1). Many carcinogens are mutagens or turn into mutagenic derivatives through biotransformation. Of these, some are known to leave a unique mutational fingerprint in the human genome (2,3). These mutational fingerprints manifest as specific types of mutations (e.g. induced base substitution/deletion/insertion), often clustering at certain nucleotide positions in cancer-related loci, in tumor genomes from carcinogen-exposed individuals (4,5). Establishing the mutational fingerprint of carcinogens is important because (i) from a mechanistic point of view, it can help infer human cancer etiology; and (ii) from a standpoint of public health, it can help reinforce hazard removal/reduction strategies for perilous environmental agents. Until recently, the mutational fingerprint of carcinogens has only been investigated in a few cancer-related genes or housekeeping genes (4). With the advent of next-generation sequencing technologies, however, a comprehensive mutational fingerprint of carcinogens can now be determined on a genome-wide scale (6,7). These breakthrough technologies are poised to survey the landscape of human cancer genome and reveal mutational fingerprints, which may be ascribed to environmental carcinogens (8). However, to verify causality, the identified mutational fingerprints need to be experimentally recapitulated in validated model systems and under strictly controlled exposure conditions (4,9).

Transgenic rodents are extensively validated model systems for establishing the mutational fingerprint of carcinogens (10). However, the mutation detection assays incorporated into these transgenic systems are only

*To whom correspondence should be addressed. Tel: +1 626 359 8111 (Ext 65918); Fax: +1 626 358 7703; Email: ania@coh.org

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amenable to conventional DNA sequencing analysis (4). This feature is prohibitive because it only allows ‘low-throughput’ detection of mutational fingerprint using direct DNA sequencing of phenotypically expressed individual mutants. Such an approach is relatively costly, extensively time consuming and extremely laborious (4). To develop a ‘high-throughput’ method for detecting the mutational fingerprint of carcinogens, we have devised a cost-, time- and labor-effective strategy, in which the widely used transgenic Big Blue® mouse mutation detection assay (Stratagene, La Jolla, CA) is made compatible with the Roche/454 Genome Sequencer FLX Titanium next-generation sequencing technology (454 Life Sciences, Branford, CT). As proof of principle, we have used this novel method to establish the mutational fingerprints of three carcinogens with varying mutagenic potencies, including sunlight ultraviolet radiation, 4-amino(biphenyl (4-ABP) and secondhand smoke (SHS) that are known to be strong, moderate and weak mutagens, respectively (11–13). For verification purposes, we have compared the mutational fingerprints of these carcinogens obtained by our newly developed method with those obtained by parallel analyses using the conventional low-throughput approach, that is, standard mutation detection assay followed by direct DNA sequencing using the capillary ABI-3730 DNA Analyzer (ABI Prism, PE Applied BioSystems, Foster City, CA). We have also performed similar analyses to establish the spontaneous mutation spectra in control (sham-treated) samples using both the new next-generation sequencing-based method and the conventional DNA sequencing.

MATERIALS AND METHODS

Selection of carcinogens and experimental treatments

To demonstrate the sensitivity and specificity of our method for detecting the mutational fingerprint of carcinogens, we chose three distinct agents with high, moderate and low mutagenic potencies, respectively. As an extensively studied environmental physical carcinogen, the ultraviolet B (UVB) fraction of sunlight ($\lambda$: 280–320 nm) is implicated in the etiology of human skin cancer and proven to be a highly potent mutagen (14–16). The aromatic amine 4-ABP is a widespread environmental contaminant, which is present in various occupational settings and tobacco smoke and considered an etiologic agent in human bladder cancer (17,18). SHS is an environmental pollutant, which is etiologically implicated in human lung cancer, and possesses relatively weak mutagenic potency (12,19).

Detailed information on the experimental treatment of Big Blue® mouse embryonic fibroblasts with UVB and the chronic exposure of Big Blue® mice to 4-ABP or SHS are provided in our previously published reports (11,13,20). Briefly, the UVB irradiation of Big Blue® mouse embryonic fibroblasts was performed in vitro at a single biologically relevant dose of 75.6 $\mu$J/cm$^2$, and under physiologic conditions (11). In vivo, 4-ABP (Sigma-Aldrich Inc., Saint Louis, MO) was administered intraperitoneally to male adult Big Blue® mice on a weekly basis for a duration of 6 weeks at increasing doses of 25–100 mg/kg bw (13). The SHS treatment of male adult Big Blue® mice was performed in vivo in exposure chambers of a TE-10 smoking machine (Teague Enterprises, Davis, CA) for 5 hr/day, 5 days/week for a duration of 4 months (20). Subsequent to all experimental treatments, genomic DNA was isolated using a standard phenol extraction-based protocol (21). The DNA was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and kept at $-80^\circ$C until further analysis.

Modification of the Big Blue® mouse mutation detection assay for compatibility with a next-generation sequencing platform

Transgenic Big Blue® rodent system is an extensively validated model for studying spontaneous or experimentally induced mutagenesis (4). The genome of these transgenic animals contains multiple copies of a chromosomally integrated $\lambda$LIZ shuttle vector, which carries two bacterial reporter genes, including the $cII$ and $lacI$ (10). To investigate the experimental induction of mutagenesis, transgenic rodents or cell cultures derived from their organs of interest are treated with a test agent in vivo or in vitro, respectively. Following a latency period needed for the expression of mutations, genomic DNA is isolated, and the $\lambda$LIZ shuttle vectors are recovered. The recovered vectors are then used in a bacterial phenotypic expression assay to identify mutants, that is, cells harboring mutations in the reporter gene(s) (10). To find the type and distribution of induced mutations in the $cII$ or $lacI$ genes, which reflect the mutational fingerprint of the tested agent, each phenotypically expressed mutant needs to be isolated individually and subjected to direct DNA sequencing. This is a time-, cost- and labor-intensive process, and as such, precludes ‘high-throughput’ generation of mutational fingerprints (4). To address this issue, we have devised a novel strategy, in which a pool of phenotypically expressed mutants, in lieu of single mutants, can be sequenced using a next-generation sequencing platform.

As the preparatory step, we performed the $cII$ mutagenesis assay on the genomic DNA of carcinogen-treated cells/mice and control (sham-treated) to phenotypically express the induced and spontaneously derived $cII$ mutants, respectively. The assay was performed using the commercially available Transpack Packaging Extract kit (Stratagene) according to the instructions of the manufacturer. Following the expression assay, 150 $cII$ mutant plaques obtained from the analysis of genomic DNA from each of the experimental or control group were cored individually and placed in a microtube containing 500 $\mu$L double-distilled water. We note that the pool of 150 mutants per sample is comparable with the number of mutants sequenced individually by the conventional low-throughput method for establishing the mutational fingerprint of carcinogens (4). For verification of reproducibility, two or more independent pools of 150 mutants were prepared from each experimental or control group simultaneously. The microtubes containing pools of 150 mutant $cII$ plaques were boiled for 5 min, and
subsequently, centrifuged at 18 000g for 5 min. Ten micro-
liters of the supernatant were immediately transferred to a
new microtube containing 40μl of a polymerase chain
reaction (PCR) mastermix in which the final concentra-
tions of the reagents were 1× PCR buffer, 1× Q
solution, 200 nM each of the forward and reverse
primers, 50 μM dNTP and 2.5U Taq DNA Polymerase
(Qiagen, Valencia, CA). The oligonucleotide primers
were custom designed to contain the forward and reverse cII
sequences (needed for amplification of the entire cII gene and its flanking regions) together with
tagged linker sequences (required for downstream appli-
cation of the Genome Sequencer FLX Titanium
next-generation sequencing). The forward and reverse
primers were 5′-cgtgagctcctgccctgcgccatcagccgtccttaca-
tccagc (tm: 72.6°C) and 5′-ctatgctcctgcacctgctcttaca-
cattcagc (tm: 72.8°C), respectively. The the-rcycling conditions were as follows: denaturation
at 95°C for 3 min, 10 cycles of amplification consisted of
45 s at 95°C, 1 min at 60°C, and 1 min at 72°C, 25 cycles of
re-amplification consisted of 45 s at 95°C and 2 min
at 73°C and finally 7 min of extension at 73°C. The PCR
amplified product (526 bp) was purified using the
QIAquick PCR purification kit (Qiagen) and kept at
−80°C until further analysis.

Genome sequencer FLX titanium next-generation
sequencing and bioinformatics data processing and
analysis

Ultradeep pyrosequencing was performed using a 454 GS
FLX (454 Life Sciences). The amplified PCR products
comprising the entire cII gene and its flanking regions
plus the 454 (A) and (B) linkers (454 Life Sciences) were
further purified by the MinElute PCR purification kit
(Qiagen). The resultant was clonally amplified on
capture beads in water-in-oil emulsion microreactors
(454 Life Sciences). The enriched-DNA beads were
deposited onto the wells of a full Roche 454 FLX
Titanium PicoTiter Plate device and pyrosequenced in
both forward and reverse directions. The 200-nucleotide
cycles were carried out in a 10-hr sequencing run, according
to the manufacturer’s instructions (454 Life Sciences).
Sequence reads were generated in FASTQ format using
the Data Processing Pipeline (v2.3) of the GS FLX System
software (454 Life Sciences). The sequence reads were
filtered by read length (within 350–550 bp limit). The filtered
sequence reads were aligned to the reference
sequence using CLCBIO Genomic Workbench’s (v4.5)
long read alignment tool. The variations of each sample
were detected using CLCBIO Genomics Workbench’s
(v4.5) SNP/DIP analysis tools, which is based on the
Neighborhood Quality Standard algorithm (22). Based
on the total number of mutants per sample, minimum
variation frequency and read number were set as thresh-
olds to detect base substitutions and insertions and dele-
tions (Indel). Because each sample contained a pool of 150
mutants, we used 0.66% (1 of 150) as minimum threshold
for detecting base substitutions or Indels. The minimum
variation frequency was also used as benchmark to calcu-
late the total number of each specific type of mutation in
each sample. For example, a 6.6% of C→T base substi-
tution in an individual sample was counted as 10 mutants
that have this type of mutation in that sample. The vari-
ation distribution was calculated based on the total
number of mutated amplicons in each sample. The vari-
atation spectrum of each sample was plotted on the refer-
ence sequence with a heatmap to improve the visualization
of variations. To minimize homopolymer sequencing
errors, which may cause assembly errors, false variations
(23) or reduced quality score of the reads (24), we imple-
mented a filtration step that uses the ‘high-quality’ read
coverage threshold and the variation status. The filter ini-
tially scans the reference sequence and identifies
homopolymer region (length: greater than or equal to 3).
Subsequently, it counts the coverage of these ‘high-
quality’ reads (Phred quality: greater than or equal to
30) relative to the variation in homopolymer region and
filters out the variations with low ‘high-quality’ read
coverage. The filter then compares the variation within
the homopolymer and at its neighboring nucleotide. If
the variation within the homopolymer is the same as
that at its adjacent nucleotide, the filter drops this variation
and considers it to have arisen from a homopolymer
sequencing error and/or assembly error. Supplementary
Figure S1 shows an example of this type of false variation
caused by a homopolymer error. In this example, there are
two variations, including A→C and C→A. The A→C
variation within the homopolymer region is followed by the
C→A variation at its neighboring base.

Statistical and bioinformatics analyses

The results are expressed as mean ± 95% confidence inter-
val. Comparison of mutant frequency data between an
experimental group and its corresponding control group
was made using the Wilcoxon rank-sum test. To determine
the reproducibility of mutation spectra established in du-
plicate/multiplicate samples analyzed by the next-gene-
ration sequencing-based method, we performed both the
hierarchical clustering analysis and the principle compo-
nent analysis (PCA) using the Partek Genomics Suite
v6.11.1116 (http://www.partek.com). To further analyze
the comparability of mutation spectra established in
duplicate/multiplicate samples analyzed by the next-
generation sequencing-based method, we performed cor-
relation analysis to calculate the similarities between the
frequency and position of each mutation detected in the
respective samples. In addition, we used correlation
analysis to compare the mutation spectra of each set of
two matching samples established by the next-generation
sequencing-based method and the conventional DNA
sequencing, respectively. We note that this correlation
analysis takes into account the similarities between the
frequency and position of each mutation detected by
the respective methods in two counterpart samples. The
applied correlation analysis uses stringent criteria to
compare two mutation spectra with respect to both the
frequency and type of each specific mutation occurred in
the entire length of the cII gene. This comparative analysis
takes into consideration the frequency and type of muta-
tions in the cII gene (as a whole) but not at certain
nucleotide positions only. So, the overall mutation frequency and pattern across the full length of the \( cII \) sequence are compared when the above correlation analysis is performed on two mutation spectra. All statistical tests were two sided. Values of \( P < 0.05 \) were considered statistically significant. The S-Plus 7.0 for Windows software (Insightful Corp., Seattle, WA) was used for statistical analysis.

**RESULTS**

**Mutant frequency and mutation spectrum**

As a potent mutagen implicated in skin carcinogenesis (11), sunlight UVB caused significant mutagenicity in Big Blue\(^{\circledast} \) mouse embryonic fibroblasts irradiated in vitro with this environmental carcinogen. The strong mutagenicity of UVB was demonstrated by a 92.6-fold increase in background \( cII \) mutant frequency from 3.01 ± 0.68 × 10\(^{-5} \) in control (non-irradiated) cells to 278.92 ± 20.63 × 10\(^{-5} \) in UVB-irradiated cells (\( P = 0.0002 \)). In vivo treatment of Big Blue\(^{\circledast} \) mice with 4-ABP, a known bladder carcinogen with moderate mutagenic potency (13), resulted in a 9.9-fold increase in background \( cII \) mutant frequency from 2.09 ± 0.20 × 10\(^{-5} \) in bladder DNA of control (solvent-treated) mice to 20.62 ± 4.77 × 10\(^{-5} \) in bladder DNA of 4-ABP-treated mice (\( P = 0.0079 \)). In vivo exposure of Big Blue\(^{\circledast} \) mice to SHS, a known pulmonary carcinogen with comparatively weak mutagenic potency (12), resulted in a 2.1-fold increase in background \( cII \) mutant frequency from 2.00 ± 0.29 × 10\(^{-5} \) in lung DNA of control (clean-air-treated) mice to 4.09 ± 0.79 × 10\(^{-5} \) in lung DNA of SHS-treated mice (\( P = 0.0011 \)).

To determine what specific type(s) of mutation have caused the significant increase in \( cII \) mutant frequency in carcinogen-treated cells/mice relative to control, we computed the absolute mutant frequency of each type of mutation in the \( cII \) gene (i.e. transitions, transversions, deletions and insertions) in the genome of carcinogen-treated cells/mice relative to control, we found the significant increase in \( cII \) mutant frequency in bladder DNA of 4-ABP-treated mice was 15.3, 40.0, 20.4, 5.7, 8.7, 1.8 and 8.1, respectively. Specifically, mutations occurring at G:C basepairs account for 81.2% of all the induced \( cII \) mutations in bladder DNA of 4-ABP-treated mice. Of these, G:C→T:A transversion mutations, which constitute 40% of all the induced \( cII \) mutations, dominate the overall increase in \( cII \) mutant frequency in bladder DNA of 4-ABP-treated mice (Figure 2A and B and Supplementary Table S1). As shown in Figure 3A and B, the percentage contributions of G:C→C:G transversions, G:C→T:A transversions, A:T→G:C transitions, A:T→C:G transversions and insertions/deletions to the overall increase in \( cII \) mutant frequency in bladder DNA of 4-ABP-treated mice were 15.3, 40.0, 20.4, 5.7, 8.7, 1.8 and 8.1 (Figure 1C and Supplementary Figure S2). The overall spectrum of induced mutations in the \( cII \) gene of UVB-irradiated cells is comparable with that previously found in the same model system using the conventional low-throughput method (\( P < 0.0001 \), Figure 1A–C, Supplementary Figure S2 and Supplementary Table S1).

Mapping of the induced \( cII \) mutations in the genome of 4-ABP-treated mice showed that the majority of mutations were located at G:C basepairs (Supplementary Figure S3). Of these, G:C→T:A transversions clustering at several codon positions in the \( cII \) gene were specific for 4-ABP treatment (Supplementary Figure S3). This spectrum of induced mutations in the \( cII \) gene of 4-ABP-treated mice is also comparable with that previously found in the same model system using the conventional low-throughput method (\( P = 0.004 \), Figure 2A–C, Supplementary Figure S3 and Supplementary Table S1).

Furthermore, mapping of the induced \( cII \) mutations in the genome of SHS-treated mice revealed that most mutations were localized to G:C basepairs (74.9%, Supplementary Figure S4). There were subtle differences between the locations of SHS-induced mutations and the spontaneously derived mutations in the \( cII \) gene in lung DNA from SHS-treated mice and control, respectively. In addition, the frequencies of mutation at certain loci along the \( cII \) gene in SHS-treated mice were slightly different from those in control. These subtle differences in the type and location of SHS-induced and control \( cII \) mutations concur with the weak mutagenicity of SHS and...
Figure 1. cII mutant frequency and mutation spectrum in UVB-irradiated cells versus control. Mutation analysis of the cII gene in mouse embryonic fibroblasts irradiated with UVB or control was performed using the cII mutagenesis assay, as described in ‘Materials and Methods’. (A) Absolute mutant frequency of each specific type of mutation in the cII gene of UVB-irradiated cells or control as determined by both the new method (NGS) and the conventional method (ABI). Average results (bars) from multiple analyses plus 95% confidence interval (error bars) are shown. (B) Percentage increase in frequency of each specific type of mutation in the cII gene of UVB-irradiated cells or control as determined by both the new method (NGS) and the conventional method (ABI). Results are expressed as ‘induced mutation (%), which is calculated as [(mutant frequency of each type of mutation in UVB group / mutant frequency of the respective type of mutation in control group / overall induced mutant frequency in UVB group / overall spontaneous mutant frequency in control group) x 100]. Distribution of mutations in the cII gene of UVB-irradiated cells (C) and control (D) as determined by the new method (NGS) and/or the conventional method (ABI).
**Figure 2.** cII mutant frequency and mutation spectrum in 4-ABP-treated mice versus control. Mutation analysis of the cII gene in bladder DNA of mice treated with 4-ABP or control was performed using the cII mutagenesis assay, as described in ‘Materials and Methods’. (A) Absolute mutant frequency of each specific type of mutation in the cII gene of 4-ABP-treated mice or control as determined by both the new method (NGS) and the conventional method (ABI). (B) Percentage increase in frequency of each specific type of mutation in the cII gene of 4-ABP-treated mice or control as determined by both the new method (NGS) and the conventional method (ABI). Results are expressed as ‘induced mutation (%)’, which is calculated as [(mutant frequency of each type of mutation in 4-ABP group / mutant frequency of the respective type of mutation in control group) \times 100] / (overall induced mutant frequency in 4-ABP group / overall spontaneous mutant frequency in control group)]. Distribution of mutations in the cII gene of 4-ABP-treated mice (C) and control (D) as determined by the new method (NGS) and/or the conventional method (ABI).
Figure 3. cII mutant frequency and mutation spectrum in SHS-treated mice versus control. Mutation analysis of the cII gene in lung DNA of mice exposed to SHS or control was performed using the cII mutagenesis assay, as described in ‘Materials and Methods’. (A) Absolute mutant frequency of each specific type of mutation in the cII gene of SHS-exposed mice or control as determined by both the new method (NGS) and the conventional method (ABI). (B) Percentage increase in frequency of each specific type of mutation in the cII gene of SHS-exposed mice or control as determined by both the new method (NGS) and the conventional method (ABI). Results are expressed as ‘induced mutation (%)’, which is calculated as [(mutant frequency of each type of mutation in SHS group / overall spontaneous mutant frequency in control group) × 100]/(overall induced mutant frequency in SHS group / overall spontaneous mutant frequency in control group)]. Distribution of mutations in the cII gene of SHS-exposed mice (C) and control (D) as determined by the new method (NGS) and/or the conventional method (ABI).
are consistent with the results found previously in the same model system using the conventional low-throughput method ($P < 0.0001$, Figure 3A–C, Supplementary Figure S4 and Supplementary Table S1).

Finally, we established the spontaneous mutation spectrum in control (sham-treated) samples using our next-generation sequencing-based method. As shown in Figure 4A and B and Supplementary Table S1, the percentages of G:C→C:G transversions, G:C→T:A transversions, G:C→A:T transitions, A:T→T:A transversions, A:T→G:C transitions, A:T→C:G transversions and insertions/deletions in the cII gene of control genomic DNA were 1.4, 3.8, 72.9, 2.5, 4.0, 5.5 and 9.9, respectively. Of these, the majority occurred at 5’-CpG dinucleotides, with G:C→A:T transitions being the predominant type of mutations (i.e. over 90% of all mutations occurring at CpG-containing sequences were G:C→A:T transitions) (Supplementary Figure S5). This spectrum of spontaneous cII mutations in the genomic DNA of control (sham-treated) cells is comparable with that previously found in the same model system using the conventional low-throughput method ($P < 0.0001$, Figure 4A and B, Supplementary Figure S5 and Supplementary Table S1). Altogether, the data indicate that our new method can sensitively and specifically detect the mutational fingerprint of three prominent carcinogens with varying mutagenic potencies, including UVB, 4-ABP and SHS, as well as establish the spectrum of spontaneous

Figure 4. Spontaneous cII mutation spectrum in control (sham-treated) mice. Mutation analysis of the cII gene in lung DNA of control mice (clean air exposed) was performed using the cII mutagenesis assay, as described in ‘Materials and Methods’. (A) Absolute mutant frequency of each specific type of mutation in the cII gene of control mice as determined by both the new method (NGS) and the conventional method (ABI). (B) Percentage of each specific type of mutation in the cII gene of control mice as determined by both the new method (NGS) and the conventional method (ABI). (C) Distribution of mutations in the cII gene of control mice as determined by both the new method (NGS) and the conventional method (ABI).
mutations in control. The levels of sensitivity and specificity of our new method are comparable with those of the currently available low-throughput method.

**Hierarchical clustering analysis and the principle component analysis**

We determined the reproducibility of the results obtained by our new method by assaying duplicate/multiplicate samples in a single run (to examine intra-assay variation) and/or in two different runs (to examine inter-assay variation). We prepared two independent pools of 150 cII mutants from UVB-irradiated cells and analyzed them in a single assay run. In addition, we used another three independent pools of 150 mutants from the UVB-irradiated cells and analyzed them in a subsequent assay run. In all cases, we verified reproducible results for the duplicate/triplicate samples analyzed in a single run as well as in two independent runs (Figure 5). More specifically, the heatmap generated by the Hierarchical Clustering Analysis, which uses the Pearson’s Dissimilarity to measure differences between frequency and position of each mutation occurred amongst different samples, showed that all the UVB-irradiated samples clustered very closely together (Figure 5A). This observation was further confirmed by the PCA mapping that showed that all the UVB-irradiated samples grouped together and remained distant from other differently treated or control samples (Figure 5B). Given the weak mutagenicity of SHS, it is also of note that the SHS-treated samples did not map too far from the control samples (Figure 5B).

We have also analyzed duplicate control samples in a single assay run as well as in a subsequent run. As shown in Figure 5, comparable results were obtained from the analysis of the above SHS-treated samples, as reflected by the clustering of all SHS-treated samples together (Figure 5A), as well as mapping of these samples closely to each other, while being apart from other differently treated samples (Figure 5B). Given the weak mutagenicity of SHS, it is also of note that the SHS-treated samples did not map too far from the control samples (Figure 5B).

**Read coverage analysis**

To demonstrate the sensitivity and specificity of our next-generation sequencing-based method and its efficient read coverage for the detection of experimentally induced/control mutations we performed a read coverage analysis on all differently treated samples and control. For each sample, we used the total number of reads as benchmark and then randomly selected $5 \times$, $10 \times$, $20 \times$, $35 \times$, $50 \times$ and $100 \times$ coverage (e.g. $5 \times$ coverage for each sample containing a pool of 150 mutants is 750 reads). Except for $100 \times$ coverage, where the full reads were used once, for all other coverage analyses ($5 \times$, $10 \times$, $20 \times$, $35 \times$ and $50 \times$), we performed the random selection of reads 2–4 times and used the average results. Note that ‘coverage’ here refers to the average number of reads sequenced per sample. For each coverage analysis, we calculated (i) the minimum true mutation, which is the percentage of mutations detected in the $x$ randomly selected reads that can also be found in the full reads, and (ii) the maximum false mutation.
which is the number of mutations detected in the x randomly selected reads that are not detectable in the full reads. As shown in Figure 6, in all cases, 20× coverage was sufficient to yield ≥97% minimum true mutation and ≤2 maximum false mutation. From 20× coverage onward, the minimum true mutation began to approach 100%, whereas the maximum false mutation started to reach negligible level.

To specifically address the effect of depth of coverage on the detection of mutation, we then grouped the true mutation ratios and false mutation counts into four categories, including (i) 5× coverage, (ii) 10× coverage, (iii) 20× coverage and (iv) 35× and plus coverage. The analysis of variance and Robust Test of Equality of Means results revealed that the depth of coverage (up until 20×) has a significant effect on the true mutation ratio, F (3, 20.53) = 20.27 (P < 0.0001) (Supplementary Table S2). Post hoc comparisons using Games-Howell’s test showed that the mean of true mutation ratio at 10× coverage (M = 0.9429, SD = 0.041) was not significantly different from that at 5× coverage (M = 0.9269, SD = 0.054) (P = 0.797), whereas this value differed significantly from that at 20× coverage (M = 0.9845, SD = 0.017) (P = 0.014). However, the mean of true mutation ratio at 20× coverage was not significantly different from that at 35× and plus coverage (M = 0.9999, SD = 0.0003). Together, these data indicate that the true mutation ratio is improved with increasing depth of coverage up until 20×, after which there is no significant improvement. Likewise, similar analysis confirmed that the depth of coverage (up until 20×) has a significant effect on the false mutation count, F (3, 28.34) = 26.55 (P < 0.0001); the false mutation count continues to reduce significantly with increasing depth of coverage up until 20×, after which there is no significant reduction (Supplementary Table S2). Altogether, these data indicate that our next-generation sequencing-based method has more than sufficient read coverage (~5 times higher than required) to detect the experimentally induced and control mutations with high sensitivity and specificity.

DISCUSSION

In this study, we have developed a high-throughput method for detecting the mutational fingerprint of carcinogens by devising a cost-, time- and labor-effective strategy in which a widely used transgenic mutagenesis assay is made compatible with a next-generation sequencing platform. Accordingly, we have modified the Big Blue® mouse mutation detection assay and incorporated it into the Roche/454 Genome Sequencer FLX Titanium next-generation sequencing technology. In addition, we have set up a detailed bioinformatics approach to process and analyze the high volume sequencing data. We have used this novel method to detect the mutational fingerprints of three prominent environmental carcinogens with varying mutagenic potencies, including sunlight UVB, 4-ABP and SHS that are known to be strong, moderate and weak mutagens, respectively (11–13). Here, we demonstrate that our new method can detect the mutational fingerprints of these three carcinogens with high sensitivity and specificity. Furthermore, we verify that the accuracy and reproducibility of this method are comparable with those of the currently available low-throughput method.

Using this new method, we have successfully established the mutational fingerprints of sunlight UVB, 4-ABP and SHS by detecting three distinct mutation spectra in the cII gene in the genomes of Big Blue® mice/cells treated with the respective carcinogens. The mutational fingerprint of sunlight UVB was characterized by the preponderance of dipyrimidine-targeted mutations, which were predominantly G:C→A:T transitions, and clustered at several codon positions in the cII gene in the genome of UVB-irradiated cells (Figure 1B and C, Supplementary Figure S2 and Supplementary Table S1). The 4-ABP-induced mutational fingerprint manifested as the prevailing G:C basepair-localized mutations, which were mostly G:C→T:A transversions, and occurred frequently at several codons in the cII gene in the genome of 4-ABP-treated mice (Figure 2B and C, Supplementary Figure S3 and Supplementary Table S1). In the case of SHS, a subtle, yet, distinguishable mutational fingerprint was established as the induced cII mutations, mostly being G:C→A:T transitions, were localized to G:C basepairs in the genome of SHS-treated mice (Figure 3B and C, Supplementary Figure S4 and Supplementary Table S1). The above-specified mutational fingerprints of these three carcinogens are comparable with those found previously in the same model system using the conventional low-throughput method (11–13).

The tested carcinogens in this study are known to induce predominantly base substitutions, a type of mutation that is effectively detectable by the Big Blue® mutation assay (10,25). The successful application of our new method for the detection of mutational fingerprint of tested carcinogens indicates that this method is suitable for establishing the mutational signature of a wide range of carcinogens. In addition, the method is flexible to be coupled with other transgenic or non-transgenic mutation detection assays if the modifications described here are implemented, accordingly. For instance, the gpt delta transgenic mutation assay, which is optimized for the detection of small/large deletion mutations and point mutations (26), can easily be incorporated into this method to allow establishing the mutational fingerprint of other classes of carcinogens, for example, clastogens. Likewise, the hypoxanthine-guanine phosphoribosyltransferase (hpri) mutation assay (27) can be introduced into this new method to offer high-throughput detection of mutational fingerprint of carcinogens in an endogenous reporter gene of the human genome.

Our overall findings show that the new method is superior to the conventional method for establishing the mutational fingerprint of carcinogens. Most importantly, the new method offers great advantages over the traditional method as it saves significant amounts of time, labor and cost. For example, the conventional method requires preparation, processing and analysis of a large number of mutants (individually), whereas the new method achieves this same objective by a single analysis of a pool of
We have calculated the amounts of time and expenses that we spent on the analysis of all our tested samples using both the conventional and the new methods. According to our calculations, the new method is approximately 20 times less time consuming and 3.5 times less costly than the conventional method. If the reduced workload of personnel is factored into these calculations, the savings will become even greater. To reduce the cost of sequencing, one can also use barcoding for multiplexing, that is, an auxiliary technique in which sample-specific barcoding adapters that include unique sequence tags and a restriction site are attached to individual samples. After pooling the tagged DNA samples, library preparation and sequencing, the tag sequences are used to identify the generated sequences that correspond to each original sample (28, 29). Currently, work in our laboratory is underway to use 12 different barcoded adapters in a single assay run, which will enable us to pool 12 independent samples together and analyze them simultaneously in each of the 8 lanes of a Roche/454 Genome Sequencer. Prospectively, the incorporation of the barcoding approach into our new next-generation sequencing-based method will save greater amounts of time, labor and cost in future sequencing projects.

As the next-generation sequencing technologies are constantly evolving and rapidly undergoing refinements, the cost of such analysis is expected to drop significantly (6–8). Due to financial constraints and lack of bioinformatics support, small laboratories may not be able to perform on-site next-generation sequencing work. Currently, however, many universities, research institutes and private companies have core facilities, which provide competitive next-generation sequencing services and bioinformatics data analysis to outside investigators. The accuracy and reproducibility of our new method, which is consistent with the known low error rate of the Roche/454 platform (30), and its comparable sensitivity and specificity with those of the existing method, together with the above-mentioned prospects are all indicative of the potential of the new method for becoming the mainstay of mutational fingerprinting for carcinogens.

Recently, Gilles et al. (31) have shown that the total error rates of the 454 GS-FLX Titanium instrument for the first 101 bases and for the full-length sequence

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**Figure 6.** Read coverage analysis for cII mutations in carcinogen-treated mice/cells versus control. For each sample, total number of reads was used as benchmark and subsequently, randomly selected 5x, 10x, 20x, 35x, 50x and 100x coverage analyses were performed as described in the text.
Thus far, few studies have used next-generation sequencing technologies for the detection of mutations in foreign DNA (using cell free environment), for example, shuttle vector or RNA template or yeast (32–34). These elegant studies have confirmed the applicability of next-generation sequencing platforms for mutagenicity analysis (32–34). However, the mutation detection systems employed in these studies may not necessarily represent some of the key determinants of mutagenesis in mammalian cells, for example, chromatin structure, DNA sequence contexts, fidelity and efficiency of DNA polymerases and DNA repair (4,35–38). In addition, these systems are not suitable for investigating organ-specific mutagenicity in relation to tumorigenesis, which is a unique property of certain carcinogens (12,13). The latter is reflective of the need for studying target-organ mutagenesis in animal models of tumorigenicity. The current literature lacks a comprehensive study, in which the application of next-generation sequencing technologies for the detection of mutations in chromosomal genes of a mammalian system is explored. Our study is the first demonstration of the applicability of these technologies for the detection of mutational fingerprint of carcinogens in a chromosomal gene in a validated mammalian model system (4,10). In spite of the increasingly popular use of transgenic model system for mutational analysis of carcinogens, the system remains low-throughput and cost, time and labor ineffective (10). This study offers a new strategy to modify the mutation detection assay in this model system by making it compatible with a next-generation sequencing platform, thus, allowing high-throughput analysis of mutational fingerprint of carcinogens in a cost-, time- and labor-effective manner.

In summary, we have developed a new next-generation-based method that can detect the mutational fingerprint of carcinogens with high sensitivity and specificity. In addition, we have shown that the accuracy and reproducibility of this new method are comparable with those of the currently available low-throughput method. Given the accuracy and reproducibility, great expediency and speed, and labor, time and cost effectiveness of this method, the method is poised to be employed in large-scale screening projects for detecting mutagenic carcinogens and become the method of choice for high-throughput DNA-sequencing analysis. Prospectively, the method will have the potential to move the field of carcinogenesis forward by allowing high-throughput analysis of mutations induced by endogenous and/or exogenous genotoxins.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Figures 1–5.

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TABLE S1. Comparison of the types cII mutations in the genome of carcinogen-treated mice/cells versus control.

| Sample ID          | G:C→C:G | G:C→T:A | G:C→A:T | A:T→T:A | A:T→G:C | A:T→C:G | Del./Ins. |
|--------------------|---------|---------|---------|---------|---------|---------|-----------|
| UVB (1st NGS)      | 2.94    | 0.00    | 87.50   | 4.41    | 0.00    | 0.00    | 5.15      |
| UVB (2nd NGS)      | 1.31    | 1.31    | 82.35   | 3.27    | 2.61    | 1.96    | 7.19      |
| UVB (3rd NGS)      | 0.00    | 0.00    | 86.11   | 2.78    | 0.00    | 1.85    | 9.26      |
| UVB (4th NGS)      | 0.00    | 0.00    | 89.89   | 2.25    | 0.00    | 2.25    | 5.61      |
| UVB (Δ NGS)        | 1.06    | 0.33    | 86.46   | 3.18    | 0.65    | 1.52    | 6.80      |
| UVB (ABI)          | 0.00    | 5.00    | 86.70   | 5.80    | 0.00    | 1.70    | 0.80      |
| Control (ABI)      | 4.10    | 12.80   | 39.20   | 6.80    | 10.10   | 11.50   | 15.54     |
| UVB (1st NGS)      | 4.00    | 0.00    | 119.00  | 6.00    | 0.00    | 0.00    | 7.00      |
| UVB (2nd NGS)      | 2.00    | 2.00    | 126.00  | 5.00    | 4.00    | 3.00    | 11.00     |
| UVB (3rd NGS)      | 0.00    | 0.00    | 93.00   | 3.00    | 0.00    | 2.00    | 10.00     |
| UVB (4th NGS)      | 0.00    | 0.00    | 80.00   | 2.00    | 0.00    | 2.00    | 5.00      |
| UVB (Δ NGS)        | 1.50    | 0.50    | 104.50  | 4.00    | 1.00    | 1.75    | 8.25      |
| UVB (ABI)          | 0.00    | 6.00    | 104.00  | 7.00    | 0.00    | 2.00    | 1.00      |
| Control (ABI)      | 6.00    | 19.00   | 58.00   | 10.00   | 15.00   | 17.00   | 23.00     |
| 4-ABP (1st NGS)    | 10.74   | 50.41   | 14.88   | 7.44    | 6.61    | 1.65    | 8.27      |
| 4-ABP (2nd NGS)    | 16.95   | 22.88   | 31.36   | 4.24    | 10.17   | 3.39    | 11.01     |
| 4-ABP (Δ NGS)      | 13.85   | 36.65   | 23.12   | 5.84    | 8.39    | 2.52    | 9.64      |
| 4-ABP (ABI)        | 11.60   | 42.20   | 24.50   | 4.80    | 4.80    | 3.40    | 8.90      |
| Control (ABI)      | 2.10    | 9.10    | 45.50   | 7.00    | 5.60    | 8.40    | 22.4      |
| 4-ABP (1st NGS)    | 13.00   | 61.00   | 18.00   | 9.00    | 8.00    | 2.00    | 10.00     |
| 4-ABP (2nd NGS)    | 20.00   | 27.00   | 37.00   | 5.00    | 12.00   | 4.00    | 13.00     |
| 4-ABP (Δ NGS)      | 16.50   | 44.00   | 27.50   | 7.00    | 10.00   | 3.00    | 11.50     |
| 4-ABP (ABI)        | 17.00   | 62.00   | 36.00   | 7.00    | 7.00    | 5.00    | 13.00     |
| Control (ABI)      | 3.00    | 13.00   | 65.00   | 10.00   | 8.00    | 12.00   | 32.00     |
| SHS (1st NGS)      | 0.00    | 9.47    | 53.68   | 6.32    | 10.53   | 12.63   | 7.37      |
| SHS (2nd NGS)      | 0.00    | 14.29   | 51.02   | 6.12    | 10.20   | 11.22   | 7.14      |
| SHS (3rd NGS)      | 8.75    | 10.00   | 58.75   | 3.75    | 10.00   | 3.75    | 5.00      |
### Table

|                | SHS (4<sup>th</sup> NGS) | SHS (Δ NGS) | SHS (ABI) | Control (ABI) | SHS (1<sup>st</sup> NGS) | SHS (2<sup>nd</sup> NGS) | SHS (3<sup>rd</sup> NGS) | SHS (4<sup>th</sup> NGS) | SHS (Δ NGS) | SHS (ABI) | Control (1<sup>st</sup> NGS) | Control (2<sup>nd</sup> NGS) | Control (3<sup>rd</sup> NGS) | Control (4<sup>th</sup> NGS) | Control (Δ NGS) | Control (ABI) |
|----------------|---------------------------|-------------|-----------|---------------|---------------------------|---------------------------|---------------------------|---------------------------|-------------|-----------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|---------------------------|
| # Mutations    |                           |             |           |               |                           |                           |                           |                           |             |           |                             |                             |                             |                             |                             |               |
| Control (ABI)  | 6.00                      | 22.00       | 188.00    | 7.00          | 13.00                     | 18.00                     | 49.00                     |                           |             |
| % Mutations    |                           |             |           |               |                           |                           |                           |                           |             |           |                             |                             |                             |                             |                             |               |
| Control (ABI)  | 2.00                      | 7.00        | 62.10      | 2.30          | 4.30                      | 5.90                      | 16.20                     |                           |             |
| # Mutations    |                           |             |           |               |                           |                           |                           |                           |             |           |                             |                             |                             |                             |                             |               |
| Control (ABI)  | 2.00                      | 7.00        | 62.10      | 2.30          | 4.30                      | 5.90                      | 16.20                     |                           |             |
| % Mutations    |                           |             |           |               |                           |                           |                           |                           |             |           |                             |                             |                             |                             |                             |               |
| Control (ABI)  | 2.00                      | 7.00        | 62.10      | 2.30          | 4.30                      | 5.90                      | 16.20                     |                           |             |
| # Mutations    |                           |             |           |               |                           |                           |                           |                           |             |           |                             |                             |                             |                             |                             |               |
| Control (ABI)  | 2.00                      | 7.00        | 62.10      | 2.30          | 4.30                      | 5.90                      | 16.20                     |                           |             |

1<sup>st</sup> NGS - 4<sup>th</sup> NGS represent the first – fourth quantifications using our newly developed next-generation sequencing (NGS) –based method; Δ NGS indicate the average results from the first – fourth quantifications.

ABI represents the conventional low throughput method using the capillary ABI-3730 DNA Analyzer (ABI Prism).

Del. = Deletion; Ins. = Insertion.
| Coverage   | N  | Mean   | Std. Deviation (SD) | Std. Error (SE) | 95% Confidence Interval (CI) | Minimum | Maximum |
|------------|----|--------|---------------------|-----------------|-----------------------------|---------|---------|
|            |    |        |         Lower Bound  | Upper Bound     |                             |         |         |
| True mutation ratio | 5x | 16 | 0.9269 | 0.05425 | 0.01356 | 0.8980 | 0.9558 | 0.83 | 1.00 |
|            |    | 10x | 0.9429 | 0.04122 | 0.01102 | 0.9191 | 0.9667 | 0.86 | 1.00 |
|            |    | 20x | 0.9845 | 0.01695 | 0.00511 | 0.9732 | 0.9959 | 0.95 | 1.00 |
|            |    | 35x and 50x | 15  | 0.9999 | 0.00026 | 0.00007 | 0.9997 | 1.0000 | 1.00 | 1.00 |
|            | Total | 56  | 0.9618 | 0.04692 | 0.00627 | 0.9492 | 0.9743 | 0.83 | 1.00 |
| False mutation count | 5x | 16 | 6.8750 | 2.65518 | 0.66380 | 5.4602 | 8.2898 | 2.00 | 11.00 |
|            |    | 10x | 3.0000 | 1.30089 | 0.34768 | 2.2489 | 3.7511 | 1.00 | 6.00 |
|            |    | 20x | 1.5455 | 0.82020 | 0.24730 | 0.9944 | 2.0965 | 0.00 | 3.00 |
|            |    | 35x and 50x | 15  | 0.9333 | 0.96115 | 0.24817 | 0.4011 | 1.4656 | 0.00 | 3.00 |
|            | Total | 56  | 3.2679 | 2.92631 | 0.39105 | 2.4842 | 4.0515 | 0.00 | 11.00 |

**Test of Homogeneity of Variances**

|                     | Levene Statistic | df1 | df2 | Significance (P) |
|---------------------|------------------|-----|-----|------------------|
| True mutation ratio | 16.928           | 3   | 52  | < 0.0001         |
| False mutation count| 10.777           | 3   | 52  | < 0.0001         |

**Analysis of variance (ANOVA)**

|                     | Sum of Squares | df | Mean Square | F   | Significance (P) |
|---------------------|----------------|----|-------------|-----|------------------|
| True mutation ratio | Between Groups | 0.052 | 3 | 0.017 | 13.039 | < 0.0001 |
|                           | Within Groups |                |                |                |                |
|---------------------------|---------------|----------------|----------------|----------------|----------------|
|                           | 0.069         | 52             | 0.001          |                |                |
| Total                     | 0.121         | 55             |                |                |                |
| False mutation count      |               |                |                |                |                |
|                           | 323.572       | 3              | 107.857        | 38.047         | < 0.0001       |
| Within Groups             | 147.411       | 52             | 2.835          |                |                |
| Total                     | 470.982       | 55             |                |                |                |

**Robust Tests of Equality of Means**

|                           | F       | df1 | df2 | Significance (P) |
|---------------------------|---------|-----|-----|------------------|
| True mutation ratio       | Welch   | 20.272 | 3   | 20.530           | < 0.0001       |
| False mutation count      | Welch   | 26.545 | 3   | 28.344           | < 0.0001       |

**Multiple Comparisons**

| Dependent Variable        | (I) Coverage | (J) Coverage | Mean Difference (I – J) | Std. Error (SE) | Significance (P) | 95% Confidence Interval (CI) |
|---------------------------|--------------|--------------|-------------------------|-----------------|-----------------|-------------------------------|
|                           |              |              |                         |                 |                 | Lower Bound | Upper Bound |
| True mutation ratio       | 5x           |              |                          |                 |                 |                |                |
|                           |              | 10x          | -0.01598                 | 0.01334         | 0.631           | -0.0514       | 0.0194       |
|                           |              | 20x          | -0.05767                 | 0.01428         | 0.001           | -0.0956       | -0.0198      |
|                           |              | 35x and 50x  | -0.07301                 | 0.01310         | < 0.0001        | -0.1078       | -0.0382      |
| Tukey HSD                 | 10x          | 5x           | 0.01598                  | 0.01334         | 0.631           | -0.0194       | 0.0514       |
|                           |              | 20x          | -0.04169                 | 0.01469         | 0.032           | -0.0807       | -0.0027      |
|                           |              | 35x and 50x  | -0.05702                 | 0.01355         | 0.001           | -0.0930       | -0.0211      |
|                           | 20x          | 5x           | 0.05767                  | 0.01428         | 0.001           | 0.0198        | 0.0956       |
|                           |              | 10x          | 0.04169                  | 0.01469         | 0.032           | 0.0027        | 0.0807       |
|                           |              | 35x and 50x  | -0.01533                 | 0.01444         | 0.715           | -0.0537       | 0.0231       |
|                           | 35x and 50x  | 5x           | 0.07301                  | 0.01310         | < 0.0001        | 0.0382        | 0.1078       |
|                           |              | 10x          | 0.05702                  | 0.01355         | 0.001           | 0.0211        | 0.0930       |
|                           |              | 20x          | 0.01533                  | 0.01447         | 0.715           | -0.0231       | 0.0537       |
|                           | Games-Howell | 5x           | -0.01598                 | 0.01747         | 0.797           | -0.0637       | 0.0318       |
|                           |              | 10x          | -0.05767                 | 0.01449         | 0.004           | -0.0984       | -0.0169      |
| False mutation count | 35x and 50x | 10x | 5x | 0.01598 | 0.01747 | 0.797 | -0.0318 | 0.0637 |
|----------------------|-------------|-----|----|---------|---------|-------|---------|---------|
|                     | 20x | -0.04169 | 0.01214 | 0.014 | -0.0760 | -0.0074 |
|                     | 35x and 50x | -0.05702 | 0.01102 | 0.001 | -0.0894 | -0.0247 |
| Tukey HSD           | 5x | 0.05767 | 0.01449 | 0.004 | 0.0169 | 0.0984 |
|                     | 10x | 0.04169 | 0.01214 | 0.014 | 0.0074 | 0.0760 |
|                     | 35x and 50x | -0.01533 | 0.00511 | 0.055 | -0.0310 | 0.0003 |
|                     | 5x | 0.07301 | 0.01356 | < 0.0001 | 0.0339 | 0.1121 |
|                     | 10x | 0.05702 | 0.01102 | 0.001 | 0.0247 | 0.0894 |
|                     | 20x | 0.01533 | 0.00511 | 0.055 | -0.0003 | 0.0310 |
|                     | 35x and 50x | 5.32955 | 0.65946 | < 0.0001 | 3.5793 | 7.0798 |
|                     | 10x | 3.87500 | 0.61617 | 0.000 | 2.2396 | 5.5104 |
|                     | 20x | 5.32955 | 0.65946 | < 0.0001 | 3.5793 | 7.0798 |
|                     | 35x and 50x | 5.94167 | 0.60512 | < 0.0001 | 4.3356 | 7.5477 |
|                     | 5x | -3.87500 | 0.61617 | < 0.0001 | -5.5104 | -2.2396 |
|                     | 10x | 1.45455 | 0.67838 | 0.153 | -0.3459 | 3.2550 |
|                     | 20x | 2.06667 | 0.62568 | 0.009 | 0.4061 | 3.7273 |
|                     | 35x and 50x | -5.32955 | 0.65946 | 0.000 | -7.0798 | -3.5793 |
|                     | 5x | -1.45455 | 0.67838 | 0.153 | -3.2550 | 0.3459 |
|                     | 10x | 0.61212 | 0.66836 | 0.796 | -1.1618 | 2.3860 |
|                     | 20x | -0.61212 | 0.66836 | 0.796 | -2.3860 | 1.1618 |
|                     | 35x and 50x | 5.94167 | 0.60512 | < 0.0001 | -7.5477 | -4.3356 |
|                     | 5x | 3.87500 | 0.74934 | < 0.0001 | 1.7972 | 5.9528 |
|                     | 10x | 5.32955 | 0.70837 | < 0.0001 | 3.3368 | 7.3223 |
|                     | 20x | 3.87500 | 0.74934 | < 0.0001 | -5.9528 | -1.7972 |
|                     | 35x and 50x | 5.94167 | 0.70837 | < 0.0001 | 3.9498 | 7.9335 |
|                     | 5x | 1.45455 | 0.42666 | 0.012 | 0.2703 | 2.6388 |
| Games-Howell        | 10x | 2.06667 | 0.42716 | < 0.0001 | 0.8878 | 3.2455 |
|                     | 5x | -5.32955 | 0.70837 | < 0.0001 | -7.3223 | -3.3368 |
|                     | 10x | -1.45455 | 0.42666 | 0.012 | -2.6388 | -0.2703 |
|                     | 35x and 50x | 0.61212 | 0.35035 | 0.323 | -0.3563 | 1.5805 |
| 35x and 50x | 5x  | 10x | 20x | 10x  | 20x |
|----------|-----|-----|-----|------|-----|
|          | -5.94167 | 0.702166 | < 0.0001 | -7.9335 | -3.2455 |
| 10x      | -2.06667 | 0.427166 | < 0.0001 | -3.2455 | -0.8878 |
| 20x      | -0.61212 | 0.35035 | 0.323 | -1.5805 | 0.3563 |
Figure S1. Schematic representation of the filtration step used in this study to minimize homopolymer sequencing errors. Detailed descriptions of how homopolymer sequencing errors are filtered out during data processing are provided in ‘Materials and Methods’.

Figure S2. Detailed mutation spectra of cII gene in UVB-irradiated mouse cells versus control. The spectrum of mutations in the cII gene of UVB-irradiated cells was determined using both the new method (A) and the conventional method (B), and the results were compared to those found in control (non-irradiated cells). The UVB-induced mutations are typed above the reference cII sequence, whereas the control mutations are typed below the reference cII sequence. For the new method, average results from duplicate samples analyzed independently in two different runs of the assay are shown. The height of mutated base represents the frequency of mutation (i.e., the higher the base, the more frequently mutated). Numbers above the mutated bases are indicatives of the percentage of mutations occurred at those specific nucleotide positions. Deleted bases are underlined. Inserted bases are shown with an arrow. Numbers below the bases are the nucleotide positions.

Figure S3. Detailed mutation spectra of cII gene in 4-ABP-treated mice versus control. The spectrum of mutations in the cII gene in bladder DNA of 4-ABP-treated mice was determined using both the new method (A) and the conventional method (B),
and the results were compared to those found in control (solvent-treated [DMSO] mice). The 4-ABP-induced mutations are typed above the reference cII sequence, whereas the control mutations are typed below the reference cII sequence. For the new method, average results from duplicate samples analyzed independently are shown (see, also legend for Fig. 2).

**Figure S4. Detailed mutation spectra of cII gene in SHS-treated mice versus control.**
The spectrum of mutations in the cII gene in lung DNA of SHS-treated mice was determined using both the new method (A) and the conventional method (B), and the results were compared to those found in control (clean-air-exposed mice). The SHS-induced mutations are typed above the reference cII sequence, whereas the control mutations are typed below the reference cII sequence. For the new method, average results from duplicate samples analyzed independently are shown (see, also legend for Fig. 2).

**Figure S5. Detailed spontaneous mutation spectra of cII gene in control mice.** The spectrum of mutations in the cII gene in lung DNA of control mice was determined using both the new method and the conventional method. The spontaneously derived (control) mutations detected by the new method are typed above the reference cII sequence, whereas those detected by the conventional methods are typed below the reference cII sequence. For the new method, average results from duplicate samples analyzed independently are shown (see, also legend for Fig. S2).
Fig. S1
Fig. S2
Fig. S3
Fig. S4
