Quantification of random genomic mutations

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Cancer cells contain numerous clonal mutations. It has been theorized that malignant cells sustain an elevated mutation rate and, as a consequence, harbor yet larger numbers of random point mutations. Testing this hypothesis has been precluded by lack of an assay to measure random mutations—that is, mutations that occur in only one or a few cells of a population. We have established a method that has permitted us to detect and identify rare random mutations in human cells, at a frequency of 1 per 10^8 base pairs. The assay is based on gene capture, by hybridization with a uracil-containing probe, followed by magnetic separation. Mutations that render the mutational target sequence non-cleavable by a restriction enzyme are quantified by dilution to single molecules and real-time quantitative PCR amplification. The assay can be extended to quantify mutation in any DNA-based organism, at different sites in the genome, in introns and exons, in unselected and selected genes, and in proliferating and quiescent cells.

The frequency of random mutations in human cells is unknown. Random mutations are mutations present in one or a few cells of a population, in contrast to clonal mutations, which are present in most or all cells of a population. Published estimates of spontaneous mutation frequencies in normal human cells have been obtained for genes that are under selective pressure both *in vivo* and during subsequent measurement. Hence, these established frequencies could be biased. Most mutation rate measurements have been carried out at the *HPRT1* (also known as *hprt*) locus because it is present as a single copy on the X chromosome. A tabulation of data for *HPRT1* indicates that the overall mutation frequency in mammalian cells varies between 10^{-5} and 10^{-7} mutations per gene or approximately 10^{-8} and 10^{-10} substitutions per DNA nucleotide[1].

Normal human cells replicate their DNA with exceptional fidelity. During every division cycle, each daughter cell receives a full and accurate complement of genetic information. Based on the assumption that the accuracy of DNA replication in stem cells is similar to that in somatic cells, we estimated that each stem cell could amass 1–2 mutant genes during 100 cell divisions in a human life span[2]. A few normal cells could contain as many as 12 mutations, based on a Poisson probability distribution. However, if the low mutation frequency observed in mouse embryonic stem cells[3] prevails in human stem cells that give rise to cancer, then normal stem cells would accumulate even fewer mutations. In contrast to normal cells, malignant cells in a tumor are markedly heterogeneous and show[4] large numbers of chromosomal abnormalities[5–7] and clonal changes in nucleotide sequence[8,9]. These changes occur in the majority of cells in the tumor and frequently involve long stretches of nucleotides (for example, in deletions and amplifications). There are no published estimates of the frequency of random point mutations, in either tumor or normal cells.

To account for the disparity between the rarity of mutations in normal cells and the large numbers of mutations in cancer cells, we hypothesized that during tumor progression, cancer cells show a mutator phenotype[10], manifested by a large number of random mutations[11]. It is these more numerous random mutations that could account for the heterogeneity of tumor cell populations, provide the substrate for evolution, and represent a definitive signature or 'footprint' of a mutator phenotype. The detection of random changes in DNA sequences has thus far been precluded by technical limitations.

Here we present a procedure to quantify the frequency of random mutation in any cell type. This mutational assay, termed the random mutation capture (RMC) method, permits us to detect mutations at a frequency of one event or fewer per 10^8 base pairs. Thus, we should be able to establish the frequency of random mutations in human cells, allowing us to evaluate the role of a mutator phenotype in carcinogenesis. Moreover, the RMC assay has broad general applicability and can be used to quantify and characterize mutation in any DNA-based organism, whether in selected or unselected genes, in exons or introns, or in quiescent or dividing cells.

RESULTS

Assay design

We have established a new, extremely sensitive method for quantifying the frequency of random mutations in a cell population (**Fig. 1**). Isolated genomic DNA is digested with several robust restriction enzymes that do not cut within the mutational target (the *Taq*I recognition sequence). The digested DNA is hybridized with an excess of probe that contains dUMP in place of dTMP and also contains a biotinylated nucleotide at the 5′ terminus. The probe is complementary to the mutational target and is synthesized by amplifying the target region in reactions containing *Taq* DNA polymerase, a 5′-biotin-terminated oligonucleotide and dUTP in

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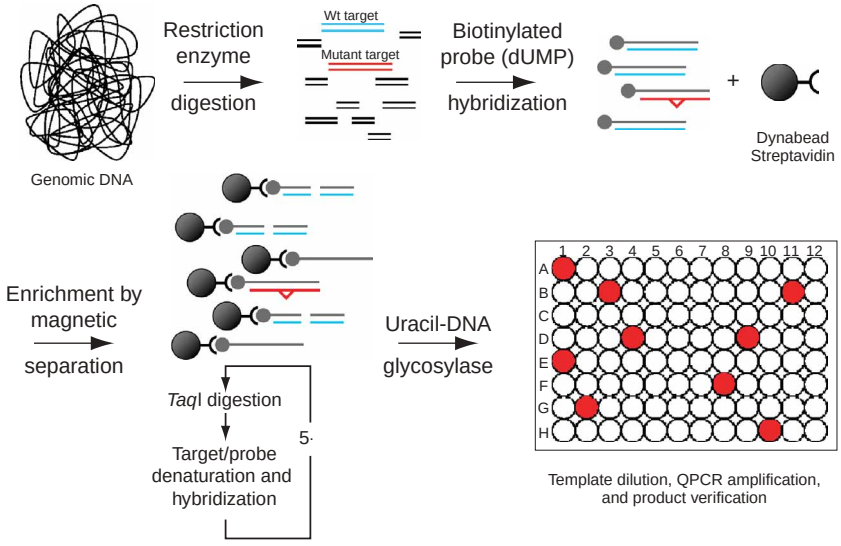

Figure 1 | Protocol for the random mutation capture assay. Blue lines represent the wild-type gene, red lines the target gene containing a mutation that renders it resistant to digestion with a restriction enzyme, and green lines the probe containing dUMP and biotin at the 5′ terminus. Red wells on the plate at bottom right contain single molecules of mutant DNA that are detected by QPCR.

place of dTTP (*Taq* DNA polymerase incorporates dUTP and dTTP with nearly identical efficiency[12]). The hybridized target is isolated by magnetic separation after complexation with streptavidin coupled to superparamagnetic polymer spheres. This enrichment of the target sequence is necessary to permit efficient digestion with the restriction enzyme *Taq*I and subsequent PCR amplification. Mutations within the mutational target are ascertained by digesting the double-stranded DNA with *Taq*I for 1 h at 65 °C; *Taq*I cleaves the wild-type sequence (TCGA) but will not digest the DNA if a mutation is present within this recognition site. We have established that the presence of dUMP in the probe does not reduce the restriction digestion efficiency of *Taq*I (data not shown). The digested product is then heat denatured at 95 °C for 1 min. The hybridization-digestion-denaturation procedure is iterated five times to ensure that mutations within the probe do not lead to false positives. For example, if the mutation frequency of the probe were 1 mutation in 10^4 base pairs (the estimated error rate *Taq* polymerase), and this step were not repeated, the frequency of false positives would be 4×10^{-4}, as a mutation at any four of the base pairs in the recognition site of the probe would prevent cleavage and genomic DNA would thus be amplified. Iteration of this procedure five times reduces the frequency of this type of false positive to 10^{-17}, as a *Taq*I sequence would have to escape digestion through five sequential hybridizations to mutant probe. After the final iteration, the probe is disabled by digestion with uracil-DNA glycosylase, abolishing its ability to serve as template in PCR, and the genomic DNA is diluted and displayed in a 96-well format. The extent of dilution is determined in preliminary experiments so that one in approximately ten wells contains a PCR-amplifiable product as measured using real-time quantitative PCR (QPCR). Based on Poisson statistics, only one in about 400 wells is expected to contain more than one amplifiable non-cleaved mutant molecule when primers are used that flank the uncut restriction site. The total number of target genes in each well is more precisely established by QPCR amplification using primers that flank regions distant from the *Taq*I restriction site. The mutation frequency is equal to the number of wells that contain a mutant sequence, divided by the total number of target base pairs screened. All positive wells recovered by use of this protocol contained a PCR product that was verified to contain a mutation, either by DNA sequencing or by resistance to *Taq*I digestion. Positive wells observed in the determination of the background mutation frequency and those required to establish the ethylnitrosourea (ENU)-induced mutational spectra were all verified by sequencing.

Mutation frequency versus mutagen dose

To detect mutations in human cells, we incubated normal human dermal fibroblasts (NHDFs) in increasing concentrations of ENU for 30 min and allowed them to undergo five rounds of doubling in the absence of ENU to allow mutation fixation. We then

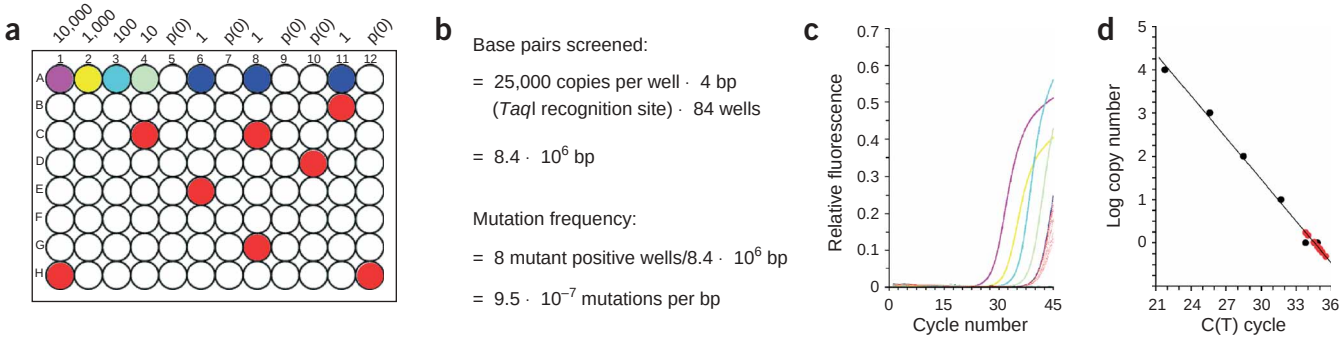

Figure 2 | Quantifying mutations in normal diploid human fibroblasts treated with ENU (500 μg/ml). (**a**) RMC assay plate array. Cellular DNA was purified and digested with *Taq*I and then diluted in 84 wells of a 96-well plate (B1 through H12) so that each well contained 25,000 copies of predigested genomic DNA. The remaining wells (A1–A12) were used in the generation of a standard curve. p(0) characterizes those wells that might have contained 1 molecule, as predicted from the Poisson equation, but did not. (**b**) Sample mutation frequency calculation. (**c**) Amplification profiles; the colors of the profiles correspond to the copy numbers in wells A1–A12 in **a**. (**d**) The standard curve illustrates that the positive mutant wells (red circles) were amplified from one molecule.

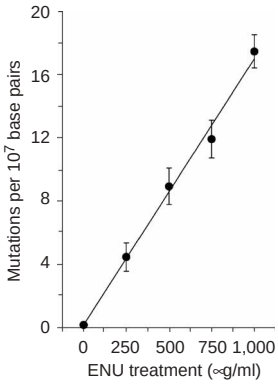

Figure 3 | Enhancement of mutagenesis as a function of ENU exposure. Normal human fibroblasts were exposed to increasing concentrations of ENU for 30 min and then incubated for 7 d in the absence of mutagen. The data show a linear increase in mutation frequency (± s.e.m.) in intron 6 in the human *TP53* gene at a *Taq*I restriction site.

determined the mutation frequency at a *Taq*I site in intron 6 of the *TP53* (*p53*) gene, as exemplified in **Figure 2** for 500 μg/ml ENU. Cellular DNA was purified and digested with *Taq*I and then diluted in 84 wells of a 96-well plate (B1 through H12) so that each well contained 25,000 copies of the target DNA segment, determined before restriction digestion as described above. The remaining wells were used in the generation of a standard curve relating copy number to the cycle at which amplification was first detected (C_T); a strict linear relationship was observed between the logarithm of copy number and the C_T value. Wells that contained an undigested copy of DNA (that is, a copy that harbors a mutation at the *Taq*I recognition site) were detected by real-time QPCR using primers that flank the site. The amplification profile of the positive, mutant wells compared to that of the standards demonstrated that single template molecules were amplified. As indicated, the mutation frequency is calculated by dividing the number of positive, mutant wells by the number of base pairs screened.

The results demonstrate that the frequency of mutation at the target sequence increases linearly with ENU doses ranging from 0 to 1,000 μg/ml (**Fig. 3**). The induced mutation frequency per base pair after exposure to 250 μg/ml ENU (4×10^{-7}) is similar to that reported in the literature[13,14]. The spectrum of substitutions that render the DNA resistant to *Taq*I includes a predominance of AT→TA and GC→AT substitutions, in accord with published results[15] (**Table 1**). Screening of 10^7 cells obtained immediately upon ENU exposure, without allowing time for mutation induction, did not yield a substantial increase in mutants over background. This result is consistent with a spontaneous mutation frequency $<10^{-7}$ and indicates that ethyl adducts interfere minimally, if at all, with digestion by *Taq*I.

Mutational target neutrality

Mutation frequencies for genes that confer a selective advantage or disadvantage may be higher or lower, respectively, than frequencies for genes that are not under selection pressure. Mutations within some introns are subject to selection and have been shown to affect cell growth[16,17]. To determine whether mutations in the target sequence confer a selective advantage or disadvantage during growth in culture, we measured mutation frequency as a function of cell divisions. NHDFs were exposed to ENU and mutation frequency was determined during growth for 2 months after the treatment (**Fig. 4**). The constancy of mutation frequency in cells that have been proliferating longer than 7 d indicates that mutations within the *Taq*I recognition site in *TP53* intron 6 are neutral, providing cells that harbor them with neither a selective advantage nor a disadvantage within the population.

Table 1 | Single-base substitutions at *Taq*I recognition site

Base substitution	Percentage
G:C→A:T	28
G:C→T:A	0
G:C→C:G	4
A:T→G:C	12
A:T→T:A	44
A:T→C:G	12

Reconstruction experiment

Reconstruction experiments were carried out to establish the effect of increasing amounts of total genomic DNA, which includes wild-type *TP53*, on mutant detection. First, a mutant *TP53* gene containing a GC→AT substitution at position 13710 in intron 6 was amplified by PCR, cloned into the pCR-XL-TOPO plasmid (Invitrogen) and transfected into *Escherichia coli*. Mutant plasmid (100 copies) was added to increasing amounts of DNA derived from NHDFs and the mutant frequency in the resulting mixtures was determined. The reconstruction experiment showed that the majority of *TP53 Taq*I restriction site mutations were recovered amid a 10^{11}-fold excess of genomic DNA (**Fig. 5**).

Background mutation frequency

To determine the background mutation frequency in human cells, we analyzed over 50 million base pairs in our mutational target sequence (located within intron 6 of *TP53*) in untreated NHDFs. Only three positive wells were observed in over 900 wells screened; the PCR-amplified product in each of the three wells contained a base substitution at the target site, as ascertained by DNA sequencing. These data indicate a background frequency of $1.6 \pm 1.2 \times 10^{-8}$ mutations per base pair and represent the first

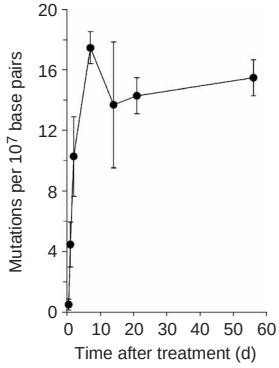

Figure 4 | Mutation target neutrality. NHDFs were treated with 1,000 μg/ml ENU for 30 min. The mutation frequency (± s.e.m.) at the *Taq*I restriction site in intron 6 of *p53* was measured as a function of time in culture after treatment.

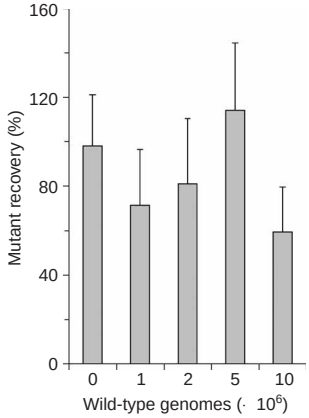

Figure 5 | Reconstruction experiment. A *TP53* gene, mutant at a *Taq*I site in intron 6, was cloned into a TOPO plasmid and amplified. One hundred copies of this construct were mixed with the indicated numbers of NHDF genomic DNA equivalents and the mutant frequency (± s.e.m.) was calculated.

estimate of the frequency of spontaneous neutral mutations in human cells. Although this is in accord with the frequency of spontaneous mutation at neutral targets in primary mouse cells[18], we cannot rule out the possibility that our estimate may represent the lower limit of detection of the RMC assay.

DISCUSSION

We have developed an extremely sensitive mutation assay to evaluate the role of a mutator phenotype in tumor progression. There are many methods available for estimating mutation frequency; however, the majority require clonal expansion and phenotypic expression of mutations[19]. These constraints limit mutation estimates to selectable markers, thus biasing measurements, and the methods cannot be applied to non-expressed portions of the genome. Moreover, only those tissues from which cells can be readily cloned can be analyzed. Genotypic selection methods (which enrich for a specific DNA sequence) circumvent many of these limitations, as they allow mutation selection at the DNA level[20]. However, the sensitivity of these DNA-based assays restricts their applicability to clonally expanded or induced mutations and has thus precluded elucidation of the spontaneous level of random mutations. Genotypic selection methods, which have used restriction enzymes, have proven to be the most sensitive. For example, the restriction-site mutation (RSM) assay[21,22] can reliably measure one mutation in a background of 100,000 wild-type molecules. However, more than ten mutant molecules are needed for mutant detection[23], limiting the assay largely to the measurement of clonal and mutagen-induced mutations. The restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR) method also relies on mutation that renders DNA resistant to digestion with restriction enzymes[24,25]. The sensitivity of the RFLP-PCR method is limited by PCR-induced mutations created during the amplification of undigested wild-type sequence by *Taq* DNA polymerase[26]. In contrast to currently available methods, the RMC assay has unprecedented sensitivity and thus allows quantification of random spontaneous mutations. Moreover, the separation and amplification of individual mutant molecules permits nucleotide sequencing and, consequently, the generation of mutation spectra.

The extremely low level of mutation in normal cells has restricted the quantification of spontaneous mutation with other assays and imparts a different limitation in the RMC assay; namely the relatively large amount of tissue required for mutation determination. Assuming that the mutation rate is 10^{-10} mutations per nucleotide per cell generation, and that the average cell undergoes 100 divisions during the lifespan of an individual, one would require approximately 1 g of normal tissue to quantify spontaneous mutation frequency. In contrast, elevated mutation frequencies greater than those in normal cells, such as we predict occur in tumor cells, should be amenable to RMC analysis with smaller amounts of tissue. Moreover, given that the assay can measure mutation at any *Taq*I recognition site, analysis of rRNA genes would considerably reduce the amount of tissue needed, as the genes that encode rRNA are each present in approximately 300 copies[27].

Many mutational assays are plagued by false positives resulting from DNA damage. This potential complication is mitigated by our finding that ethyl lesions interfere little, if at all, with cutting by *Taq*I and do not seem to increase mutagenesis. Thus, treatment of cells with 1 mg/ml ENU for 30 min, followed by immediate DNA isolation without time for repair (**Fig. 4**, time 0), yielded a mutation frequency consistent with the background level of mutation. Nonetheless, the DNA we isolated could contain small endogenous lesions other than ethyl adducts, and some might prevent *Taq*I cleavage, induce base-pairing errors during PCR-amplification, and be scored as mutations. The most prevalent endogenous damage would probably be abasic sites and other small lesions resulting from damage by reactive oxygen species or alkylating agents[28]. However, the lack of discernible interference by ethyl adducts in the RMC assay, and by 4-nitroquinoline 1-oxide adducts in *Taq*I digestion[23], suggests that oxidative lesions[29] and abasic sites[30] which are not efficiently bypassed by *Taq* DNA polymerase will, likewise, not affect mutation measurements. Indeed, the fact that the mutation frequency we observe in human diploid fibroblasts is 10^{-8} argues strongly that the presence of small endogenous pre-mutagenic alterations is not a major consideration. Finally, lesions that prevent amplification by *Taq* polymerase, such as large blocking adducts, would not be scored as mutations.

In summary, we have established a non-clonal mutational assay for the measurement and characterization of neutral spontaneous mutation. We present the first measurement of the frequency of unselected human mutations, of 1.6×10^{-8} per base pair. We expect that the sensitivity of the RMC assay will be exploited to evaluate the role of mutation in cancer, by measuring random mutation accumulation in normal human tissues and tumors. Although this assay was developed to study mutation accumulation in cancers, it can also be used to study mutation frequencies in other disciplines of biology. Areas of interest include measurements of mutation frequencies in different DNA contexts, in different tissues, during development, in different cell types, in different individuals, in genetic syndromes and in individuals exposed to genotoxic agents. Analysis can be extended to additional restriction enzyme recognition sequences by using other restriction endonucleases that have the favorable properties of *Taq*I. Thus, the assay has the potential for broad general applicability and may facilitate the study of mutation accumulation in various cells at restriction sites throughout the genome.

METHODS

Enzymatic target preparation. The mutational target was made accessible to hybridization and subsequent enrichment by digestion with restriction enzymes. First, 120 µg of genomic DNA was digested with 1 µl *Pvu*II (100 U) and 1 µl *Rsa*I (100 U), which flank the mutational target, as well as 1 µl *Eco*RI (200 U), 1 µl *Eco*RV (200 U) and 1 µl *Bam*HI (200 U) (all enzymes from New England Biolabs) that cut in other segments of the genome. The reaction was carried out in 500 µl and also contained 5 µg BSA and 20 mM NaCl, 50 mM Tris-HCl (pH 7.9), 2 mM $MgCl_2$ and 0.2 mM dithiothreitol. Digestion was at 37 °C for 16 h. The reaction was then transferred to a Microcon YM-50 tube (Millipore), centrifuged for 12 min at 12,000*g*, and washed twice with 400 µl of 1 M NaCl by centrifuging at 12,000*g* for 12 min and then discarding the supernatant each time. The Microcon tube was then inverted and centrifuged at 1,000*g* for 3 min and the released DNA diluted in 500 µl 1 M NaCl.

Probe synthesis and hybridization. A 909-bp DNA complementary to the mutational target area of the transcribed strand of the *TP53* gene containing intron 6 was generated with the following primers: 5′-biotin-CAT CAT ACA GTC AGA GCC AAC CTA GG-3′ (reverse) and 5′-CTG TGG GTT GAT TCC ACA CC-3′ (forward). The 50-µl reaction contained 0.05 U/µl JumpStart AccuTaq DNA polymerase (Sigma), 50 mM Tris-HCl (pH 7.9), 15 mM ammonium sulfate (pH 9.3, adjusted with NH_4OH), 2.5 mM $MgCl_2$, 1% Tween 20, 1 µl ACGU dNTP mix (Sigma) and 100 ng of the appropriate genomic template. PCR amplification was carried out as follows: initial denaturation at 96 °C for 1 min followed by 35 cycles of 94 °C for 15 sec, 60 °C for 30 sec and 68 °C for 1.5 min. Samples were maintained at 70 °C for 5 min and stored at 4 °C. All amplification products were verified by agarose gel electrophoresis and then purified with a QIAquik PCR Purification Kit (Qiagen). Next, 20 µl of the purified biotinylated DNA fragments was resuspended with 50 µg prewashed Dynabeads M-280–Streptavidin and 20 µl Kilobase binding solution (Dynal Biotech) and incubated at room temperature for 3 h on a roller. (The washed Dynabeads must be used immediately and should not be stored.) The solution was then placed in the Dynal Magnetic Particle Concentrator (MPC) (Dynal Biotech) and the supernatant removed. The Dynabead-DNA complex was washed twice in 40 µl washing solution (10 mM Tris-HCl, 1 mM EDTA, 2.0 M NaCl) and resuspended in 50 µl of 10 mM Tris-HCl (pH 7.9). The sample was incubated at 100 °C for 5 min, immediately placed in the MPC, washed with 500 µl 1 M NaCl and resuspended in 100 µl 1 M NaCl.

Target gene capture and genotypic selection. Digested genomic DNA (500 µl) was added to 10 µl of Dynabead-Streptavidin probe, boiled for 5 min, rapidly cooled on ice and incubated at 60 °C for 16 h rotating in a micro hybridization incubator. (The efficiency of target recovery is improved when the probe is synthesized immediately before hybridization.) The target DNA was purified using the MPC, washed twice with 100 µl of 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and then resuspended in 100 µl of the same solution, supplemented with 10 µg BSA and 100 U of *Taq*I (New England Biolabs). Digestion was carried out at 65 °C for 1 h, heated to 95 °C for 1 min to allow for DNA denaturation and then incubated at 50 °C to reanneal the probe-template complex. This entire protocol was carried out five times with 100 U of *Taq*I added per cycle. After each *Taq*I addition, samples were thoroughly mixed by pipetting and briefly centrifuged before the next incubation to ensure efficient digestion. When the digestion procedure was complete, 10 U of uracil-DNA glycosylase (UDG) in 95 µl of 50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol was added to catalyze the release of uracil from the uracil-containing probe. Incubation was for 2 h at 37 °C.

Mutation detection. Reaction mixtures (25 µl) contained Brilliant SYBR Green QPCR master mix (Stratagene), 0.5 U UDG, 25,000–100,000 *Taq*I -predigested mutational targets and 1 µM each of the following flanking primers: forward *TP53* target intron 5′-CTC TGG GAG GAG GGG TTA AG-3′ and reverse *TP53* target intron 5′-CCC GAG TAG CTG GGA CTA CA-3′. In wells used to generate standard curves, the control reverse primer (5′-TCC CAA CCT CGT GAT CCG CCT-3′) upstream of the *Taq*I restriction site was used in place of the reverse *TP53* target intron. To amplify the aforementioned fragments, thermal cycling was carried out using the Opticon 2 system (MJ Research). The samples were amplified as follows: UDG incubation at 37 °C for 10 min, an initial denaturation step at 95 °C for 2 min, and then 50 cycles of 95 °C for 30 sec, 60 °C for 1 min and 72 °C for 1.5 min. Samples were held at 72 °C for 5 min and immediately stored at −20 °C. The DNA product from all PCR positive wells was either (i) incubated with *Taq*I and verified, by agarose gel electrophoresis, to be insensitive to digestion or (ii) sequenced to identify the mutation at the *Taq*I recognition site. All experiments were performed in triplicate and no false positives were recovered in this work.

DNA isolation. Cells were pelleted and resuspended in proteinase K solution (2 mg/ml; Sigma). Genomic DNA was purified from the cell suspension after an incubation time of 2 h at 55 °C, followed by phenol/chloroform (1:1, vol/vol) extraction and precipitation with ethanol. The precipitated DNA was spooled onto a hooked glass Pasteur pipette, air dried and dissolved in 50 µl Tris-HCl (pH 8.0). The DNA was allowed to dissolve overnight at room temperature and its concentration was then determined spectrophotometrically.

Cell culture. Neonatal normal human dermal fibroblasts (Cambrex) were cultured in DMEM (Gibco-BRL) containing 10% (vol/vol) FBS (HyClone), 1% L-glutamine and 1% penicillin-streptomycin (Gibco). An atmosphere of 5–7% CO_2 was maintained in a humidified incubator at 37 °C.

Mutagen treatment. All cultures were prepared by seeding 1.5×10^6 cells in 100-mm culture dishes and incubating under standard growth conditions. At 24 h after plating, cells were treated with ENU (0–1,000 µg/ml; Sigma) for 30 min in serum-free medium under standard cell culture conditions. Cultures were then washed three times with PBS and incubated as described above. Cells were harvested by trypsinization 0, 7, 14, 21 and 56 d after treatment, pelleted by centrifugation (1,000*g*), quick-frozen with liquid nitrogen and stored at −80 °C until further analysis.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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