

Adduction of Human *p53* Gene by Fecal Water: An *In vitro* Biomarker of Mutagenesis in the Human Large Bowel

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Abstract

A polymerase arrest assay was designed to determine sites of adduction in the human *p53* gene induced by incubation with fecal water. Significant formation of adducts was observed on *p53* DNA after a 2-h incubation in fecal water from 10 of 17 samples studied. Large sample-to-sample variation was ob-

served. The major sites of polymerase termination occurred at nucleotides 3' to guanine residues. Adduct sites coincided with colorectal cancer *p53* mutation "hotspots," highlighting the potential carcinogenicity of fecal material. (Cancer Epidemiol Biomarkers Prev 2007;16(12):2681–5)

Introduction

The majority of colorectal cancer incidence is sporadic with lifestyle factors such as diet, exercise, body weight, smoking, and alcohol having an important influence (1). It is thought that contact with the fecal stream is a major mechanism inducing somatic mutations (2). Bruce et al. (3) first brought to light the mutagenic capacity of human feces, which is known to contain a variety of potentially genotoxic substances including fecapentaenes, bile acids, heterocyclic amines, polycyclic aromatic hydrocarbons, and *N*-nitroso compounds measured as apparent total nitroso compounds (ATNC; refs. 4, 5). However, there have been few studies characterizing the nature of these diverse genotoxins. Previous studies have measured levels of DNA strand breaks induced by fecal water using the comet assay (5-7) and we have now investigated whether this material is capable of inducing DNA adducts characteristic of these classes of agents.

Methods used to determine adduct formation include ³²P-postlabeling analysis, immunochemical methods, fluorescence techniques, and mass spectrometry analysis, which are all labor-intensive, time-consuming techniques (8). ³²P postlabeling is currently thought to be the best method as it is extremely sensitive and can be applied to complex mixtures. However, it is time-consuming and involves the use of relatively high levels of radioactivity. The polymerase arrest assay is a relatively sensitive, quick, and simple method for the semiquantification and sequence localization of DNA adduction (9).

In this study, the mutagenicity of fecal water was determined using a polymerase arrest assay that allowed the localization of adducts that blocked DNA replication of a human *p53* gene sequence. Several large databases of tumor-associated *p53* mutations already exist (10, 11). Thus, sites of adduction from genotoxic agents can be compared with positions of known colorectal cancer mutations (12). To our knowledge, this is the first study to undertake adduct mapping in *p53* DNA after fecal water incubation.

Materials and Methods

Sample Preparation. Seventeen samples of fecal water were extracted from available fecal samples from 10 volunteers taking part in controlled dietary studies as previously described (5, 13). Aliquots from defrosted fecal samples that had been snap frozen on dry ice were homogenized for 2 min and then centrifuged at 50,000 × *g* for 2 h. Aliquots of the supernatant fecal water were stored at –80°C before analysis.

PCR Generation of *p53* Template DNA. Plasmid pLS76 (14) containing the wild-type human *p53* sequence was used (5 ng/reaction) to generate an amplicon spanning most of the DNA-binding domain of the *p53* gene, comprising codons 126 to 316. PCR conditions were as follows: 95°C for 5 min, then 29 cycles of 95°C for 1 min, 65°C for 1 min, 72°C for 1 min, followed by 2 min at 55°C, then 5 min at 72°C. Primers were as follows: forward, 5'-TACTCCCCTGCCCTCAA-CAAG-3'; reverse, 5'-GGAGAGGAGCTGGTGTGTT-3'. One tenth of each PCR reaction was checked for size (expected size ~570 bp), and yield on a 2% agarose gel. The resulting *p53* amplicon was attached to streptavidin-coated magnetic beads (Dynabeads, Dynal) via the biotinylated forward primer.

DNA Template Incubations with Fecal Water. Fecal waters were defrosted, and 100 μL of each sample was centrifuged in an Ultrafree-MC centrifugal filter unit

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(5,000 NMWL, Biomax), at $5,000 \times g$, for 45 min. Fifty microliters of each filtrate was placed into a separate well of a 96-well plate, and 10 μL ($\sim 5 \mu\text{g}$) of DNA-Dynabead template attached to Dynabeads (DNA-Dynabeads) were added to each before being rotated while being incubated at 37°C using an Eppendorf Thermomixer Comfort (Eppendorf). Samples were incubated for 2 h before being placed on a magnet and washed thrice with 50 μL of TE buffer. There was no significant increase in adduction with incubation periods above 2 h (data not shown).

Polymerase Arrest Assay. Sites of adduction in the *p53* DNA caused by incubation with fecal water samples were characterized using a polymerase arrest assay. First, ssDNA template (sense strand) was produced by incubating the DNA-Dynabead complex in 0.1 mol/L sodium hydroxide and then isolating the streptavidin/biotinylated strand using a magnet. A fluorescent primer PSA3 (Hex-labeled 5'-TGGGCAGTGCTCGCTTAGTG-3') was used for sequencing codons 200 to 300. The labeled primer (3 $\mu\text{mol}/\mu\text{L}$) was annealed to treated DNA-Dynabead template (2.5 μg) in 10 μL Sequenase buffer (Tris-HCl 40 mmol/L, MgCl_2 20 mmol/L, NaCl 50 mmol/L) by heating at 60°C for 10 min, followed by cooling at room temperature for 15 min. Seven units of Sequenase Version 2.0 (USB; Amersham Biosciences) was added to the primer annealing reaction in an 8 μL deoxynucleotide triphosphate mix (1.25 mmol/L), and the reaction was incubated at 37°C for 10 min. The supernatant was removed and the DNA-Dynabead template washed in TE (Tris-HCl 10 mmol/L, EDTA 1 mmol/L) before being resuspended in loading buffer (5 μL ; formamide/EDTA 5:1). A TAMRA500 fluorescent size marker (0.5 μL ; Applied Biosystems) was added before denaturation of each sample at 90°C for 3 min. The products were electrophoresed for 2.5 h in an acrylamide gel (GenePage 4.25%, NBS Biologicals Ltd.) using an Applied Biosystems 377 Sequencer. Sequencing reactions were done on untreated DNA-Dynabead template using the same procedure, but with the deoxynucleotide triphosphate mix replaced with ddA or ddG sequencing mix to provide a fragment size ladder (Sequenase Version 2.0).

Applied Biosystems Genescan Analysis software was used for the analysis. Standard size markers were included to allow for lane-to-lane variation in the gel run. Fluorescent intensity (peak height) of a particular

arrest site was used as a semiquantitative indicator of the level of damage. A negative control was run on every gel (DNA incubated with TE buffer only). Fluorescent peaks were considered to reflect sites of adduction if they were more than double that of the corresponding negative control.

UVC Dose Response Validation. To provide validation of the polymerase arrest assay and to show a dose-response relationship, *p53* template was prepared as described above and $\sim 2.5 \mu\text{g}$ of the DNA-Dynabead complex were exposed to UVC light (254 nm, 0.7 mW/cm^2) for 2, 4, or 6 min. The polymerase arrest assay was then conducted as previously described using primer PSA3. Sites of UVC-induced polymerase arrest within the *p53* sequence (sense strand, codons 241-290) were determined.

Results

UVC Dose Response. UVC light was used to show that damage can be localized within the expected sequence context accurately and quantitatively using the polymerase arrest assay (Fig. 1). A clear dose response with respect to polymerase inhibition is visible at multiple sites, with levels of adduction increasing with respect to duration of UVC exposure. Furthermore, examples of a dose-response effect are seen exclusively at potential sites of pyrimidine dimer formation.

Fecal Water Incubations. To successfully recover DNA, it was found necessary to microfilter fecal water samples before incubations to remove factors capable of degrading DNA. ATNC levels were measured before and after filtration, which showed that $\sim 25\%$ of ATNC was removed via the filtering step (results not shown).

Significant formation of polymerase progression inhibiting adducts within codons 211 to 291 (nucleotides 13393-14542) was observed with 10 of the 17 samples (Fig. 2). These 10 samples came from 6 of the 10 volunteers, and results from samples obtained on different days and diets (a, b, c) from subjects 2, 3, and 4 were similar. In total, 104 different sites of polymerase termination were found within this region ($>1/3$ of the available nucleotide sites), with considerable variation in the level of adduction and the number of samples inducing adducts at each site. No obvious base specificity

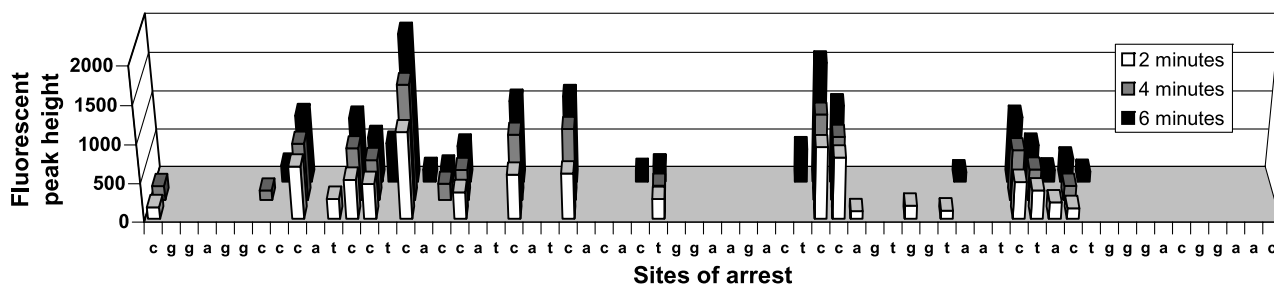


Figure 1. Sites of polymerase arrest after exposure of *p53* DNA (codons 248-268) to 2, 4, and 6 min of UV light (254 nm), showing dose response at sites where arrest occurred. Peak height (fluorescent intensity) is proportional to the amount of DNA damage at each site.

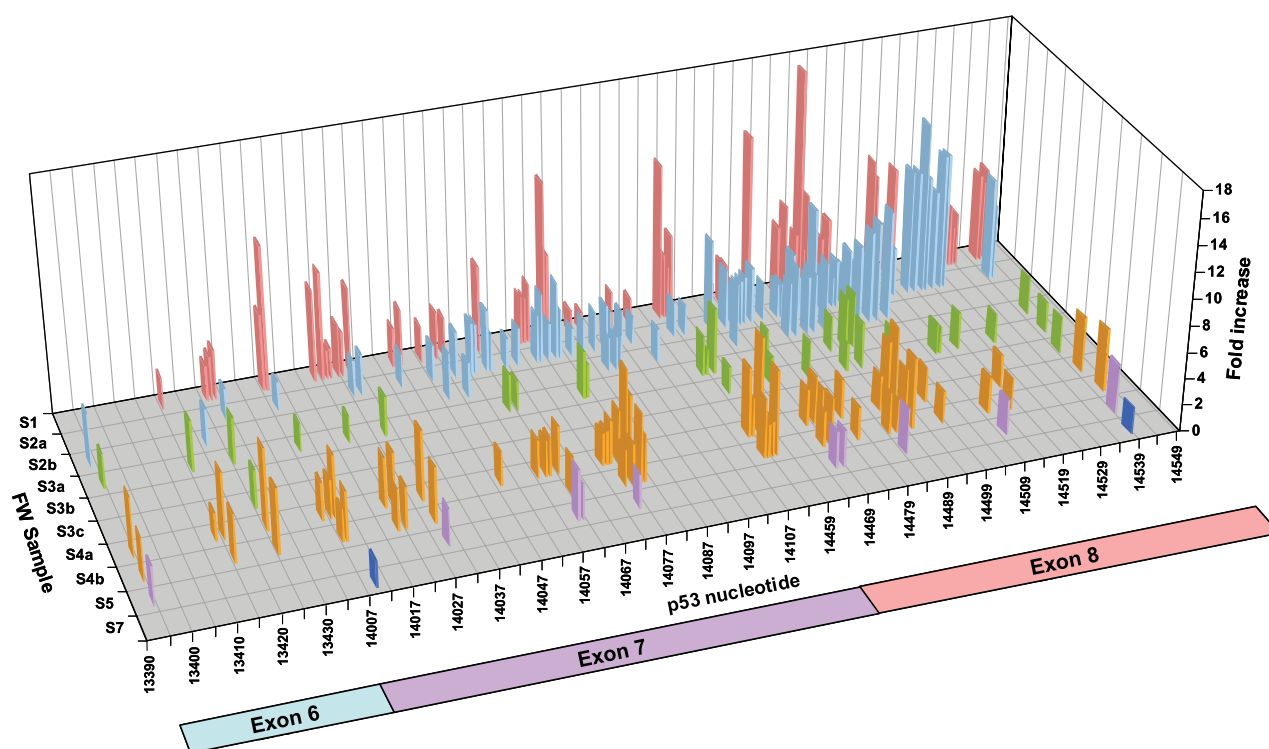


Figure 2. Sites of polymerase arrest within the *p53* cDNA sequence, codon 210 (nucleotide 13390) to codon 294 (nucleotide 14549), after treatment with 10 fecal water samples from six individuals (S1-S7). Peak height represents the fold increase in level of polymerase arrest above the negative control. The position of the arrest sites relative to exons 6 to 8 is shown. Nucleotide numbering is taken from the IARC *p53* database (10). Samples from the same individual are colored similarly.

was apparent when sites of polymerase termination were examined; 36 of the 104 termination sites were located opposite cytosine bases, 29 at guanines, 24 at thymines, and 15 at adenines.

It is interesting to note that twice as many termination sites occurred at nucleotide positions prior (3') to guanine residues (42 of 104), compared with adenine, cytosine, and thymine residues (20, 21, and 21, respectively). This represents 58% of the guanine bases within the 252 bp sequence, 39% of thymines, 32% of cytosines and 33% of adenines. This is consistent with preferential adduction of guanine residues leading to premature termination on the 3' flanking nucleotide, as has been noted with primer extension studies of site-specific adducted oligonucleotides (9).

There were no clear polymerase arrest hotspots within the targeted sequence. Clusters of multiple termination sites occurred at codons 249/251 (nucleotides 14072-14080 in Fig. 2), codons 261/262 (nucleotides 14452-55), and codons 272 to 275 (nucleotides 14484-14492). The most frequently adducted site (7 of 10 samples) was found at codon 273 (nucleotide 14486). A comparison of the distribution of all the arrest sites with total mutation sites observed in the same region of *p53* in human colorectal tumors (IARC version 10 database; ref. 10) revealed no obvious similarities. However, when a comparison was made between mutated sense-strand guanines in colorectal tumors, with guanines found on the 5' side of polymerase arrest sites, a clear correlation

was seen. Of the 40 mutation sites in human colorectal tumors involving sense-strand guanine nucleotides between codons 210 and 294, 27 seem to be adducted by one or more of the fecal water samples in this study (Fig. 3). In addition, four of the five most frequently mutated guanines were adducted by one or more fecal water samples. Overall, significantly more mutations occur at fecal water adducted guanines than would be expected by chance ($P < 0.001$), taking into account the frequency of guanine nucleotides within the target sequence. For this comparison, sites of mutation at CpG sites were omitted.

Discussion

Knowledge of adduct formation caused by the multitude of substances present in fecal samples is limited, and the main aim of this study was to map adduction in the *p53* gene induced by fecal water constituents and compare these sites with those of mutations in colorectal cancer. We were also able to determine whether the assay would provide a reliable and feasible alternative to current adduct detection methods.

Fecal water was found to generate polymerase arrest at all four bases, but principally at cytosine residues (32% of adduct sites). However, 40% (42 of 104) of polymerase arrest sites occurred opposite nucleotides on the 3' flanking side of guanines, which is consistent with

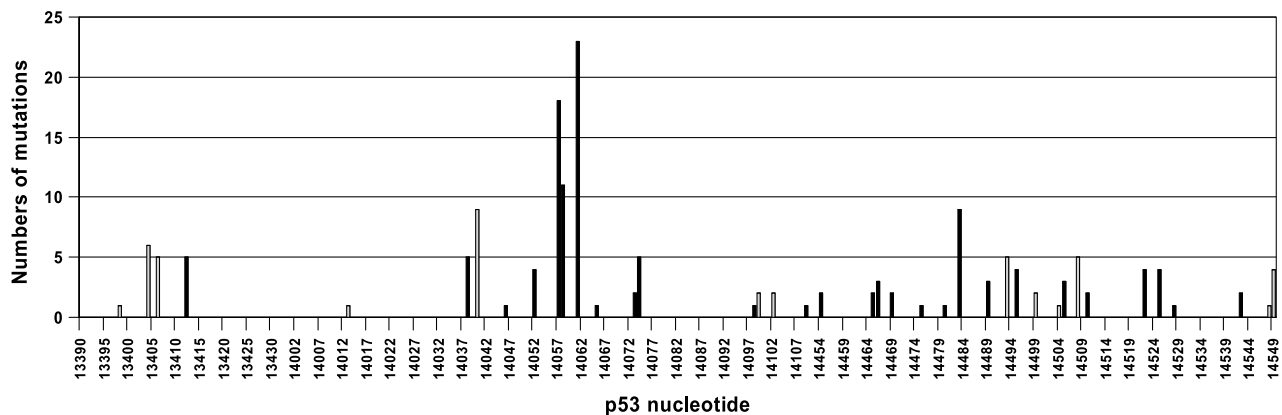


Figure 3. Comparison of distribution of sites of adduction induced by fecal water and mutations in colorectal tumors. Adducts and mutations at guanines within the sense strand from codons 210 to 295 are used for the comparison. Columns, sites and numbers of mutations taken from the IARC *p53* mutation database (10). Black bars, sites of mutation that coincide with sites of adduction. Nucleotide numbering is taken from the IARC *p53* database (10).

extension-blocking lesions being present on the guanine residues (9). Most genotoxins, particularly *N*-nitroso compounds, have been found to preferentially react with guanine, and relatively few show specificity for pyrimidines (15). This leads us to conclude that the preferential site of adduction in this study was at guanine residues and that fecal water is capable of inducing polymerase extension-blocking lesions.

The fluorescent intensity of the truncated products generated in the polymerase arrest assay reflects the frequency of adducts in the treated template DNA. Interestingly, the level of polymerase inhibition induced by fecal water in these experiments was comparable with the levels generated by 2-min exposure to UVC under the conditions described (data not shown). This suggests that in some instances, fecal water can contain significant and easily detectable levels of genotoxic factors. A comparison of polymerase arrest hotspots and human *p53* mutation hotspots (10) reveals no direct correlation in terms of levels of adduction and numbers of mutations. This observation is consistent with the role of other factors, such as chromatin structure, DNA repair, and polymerase fidelity, in the determination of mutational hotspots. However, it would seem that there is a clear correlation between the distribution of adduct sites and mutation sites, especially when this comparison is restricted to mutated and adducted guanines (Fig. 3). Four of the five largest hotspots for guanine mutations were shown to be adducted by fecal water samples, and significantly more mutations correlate with adducted guanines than would be expected by chance. The results of this preliminary study therefore suggest that *p53* adducts induced by fecal water incubation occur at sites that play an important role in colorectal carcinogenesis.

The predominant DNA adducts formed during metabolism of nitrosamines are *N*⁷ and *O*⁶-alkyl-guanine adducts, and other methylated versions. Heterocyclic amines (such as IQ, MeIQx, and PhIP) are also known to form bulky aromatic adducts [e.g., *N*-(deoxyguanosin-8-yl)-IQ, *N*-(deoxyguanosin-8-yl)-

PhIP] found usually on G-C base pairs. Similarly, polycyclic aromatic hydrocarbons cause bulky adducts on guanine sites. In the present study, we have shown that polymerase extension is clearly inhibited, possibly by potent bulky adducts produced by genotoxins present in fecal water.

We were unable to assess the effects of diet or endogenous nitrosation (measured as ATNC) in this study due to the limited numbers of samples available following published work from previous studies (5, 13), which made statistical comparisons between different dietary periods and different individuals non-viable. Nevertheless, results from samples obtained on different days and diets (a, b, c) from subjects 2, 3, and 4 were similar (Fig. 2), which suggest that the technique is repeatable. It was necessary to use fecal water rather than fecal homogenates, because of the particulate nature of fecal homogenate that limits its use in genotoxicity assays. Fecal water fraction has been reported to be more efficient than fecal solids at altering the growth characteristics of colonocytes rather than homogenate (16, 17) and has been used for genotoxicity assays such as the comet assay (6, 18, 19). However, fecal homogenates have been shown to contain 3 times more ATNC and 100 times more nitrosated heme than fecal water (5, 20). Furthermore, there was a 25% loss of ATNC in the necessary fecal water filtering step. Previous assays using fecal water have investigated effects on intact whole cells such as in the *Salmonella typhimurium* genotoxicity assay and HT29 cells in the comet assay, where systems for repair of DNA damage remain intact (3, 5, 18). Although the present system may not represent effects *in vivo*, it nevertheless shows the potential for mutagenesis and thus initiation.

In summary, the polymerase arrest assay was successful in detecting adduction of the *p53* gene after fecal water incubation. The results of this preliminary study are consistent with the hypothesis that fecal matter is potentially genotoxic and capable of significant DNA damage, which, judging by the positions of damage noted, could give rise to mutations.

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