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***In vitro* recapitulating of *TP53* mutagenesis in hepatocellular carcinoma associated with dietary aflatoxin B₁ exposure**

Ahmad Besaratinia^{1,*}, Sang-in Kim¹, Pierre Hainaut², and Gerd P. Pfeifer¹

¹ Department of Cancer Biology, Beckman Research Institute of the City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010, USA

² International Agency for Research on Cancer, 150 Cours Albert Thomas, 69372 Lyon, Cedex 08, France

Abstract

Background & Aims—Dietary exposure to aflatoxin B₁ (AFB₁), in addition to other known factors, increases risk for human hepatocellular carcinoma (HCC). HCCs from AFB₁-exposed individuals frequently have distinct *TP53* mutations, such as G to T transversions in the 2nd guanine of codon 249 (AGG to AGT), and a characteristic mutational spectrum predominated by G:C to T:A mutations.

Methods—To recapitulate the distinctive features of *TP53* mutations in AFB₁-associated HCC, we investigated AFB₁-induced DNA adduction in relation to mutagenesis in transgenic mouse fibroblasts exposed to AFB₁ *in vitro*.

Results—Immuno-dot-blot determination of DNA adducts in the overall genome of AFB₁-exposed cells revealed the dose-dependant formation of persistent imidazole ring-opened AFB₁-DNA adducts. DNA footprinting analysis of the *cII* transgene in AFB₁-exposed cells verified the dose-dependent and sequence-specific formation of DNA adducts. Preferential formation of AFB₁-induced DNA adducts along the *cII* transgene was almost exclusively localized to guanine-containing sequences encompassing CpG dinucleotides. Mutation analysis of the *cII* transgene in AFB₁-exposed cells revealed a dose-dependent induction of *cII* mutant frequency ($P < 0.001$) and a unique induced mutational spectrum characterized by predominant induction of G:C to T:A transversions that occurred within CpG sequence contexts. Notably, codons 42 and 45 of the *cII* transgene, which have identical sequence contexts to that of codon 249 of human *TP53*, constituted 2 frequently mutated sites in AFB₁-exposed cells that contain the G to T transversion signature mutation at their 3rd base positions.

Conclusions—In this model system, AFB₁-induced DNA adduction and mutagenesis recapitulate the unique mutational features of *TP53* in AFB₁-associated human HCC.

*Correspondence to: Tel: (626) 359 8111 ext: 65918; Fax: (626) 358 7703; E-mail: ania@coh.org.

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Introduction

Human hepatocellular carcinoma (HCC) is the 5th most common malignancy and the 3rd most frequent cause of cancer-related death, worldwide ^{1–3}. Cancer epidemiology investigations have implicated a multifaceted etiology for human HCC ^{4, 5}. In addition to chronic infections with hepatitis B and hepatitis C viruses, and lifestyle choices, *e.g.*, alcohol abuse, dietary exposure to aflatoxin B₁ (AFB₁) is a major risk factor for development of human HCC ^{1–5}. The etiologic involvement of AFB₁ in human HCC can be inferred from the high incidence of this disease in certain geographical regions, *e.g.*, Southeast Asia and sub-Saharan Africa, where warm and humid climate promotes mold growth and contamination of food supplies by this naturally occurring mycotoxin ^{4, 6}. A substantial number of HCC tumor samples harbors a unique mutation in the *TP53* tumor suppressor gene, *i.e.*, G to T transversion in the 2nd guanine of codon 249 (AGG to AGT), and a characteristic *TP53* mutational spectrum predominated by G:C to T:A mutations (*see* Fig. 1a, b) ⁷. Strikingly, this signature of *TP53* mutations is highly augmented in HCC tumor samples from individuals residing in areas where contamination of foodstuffs by AFB₁ is prevalent (*see* Fig. 1c, d) ^{8–11}.

The carcinogenicity of AFB₁ is partly ascribed to its ability to induce DNA damage (adduct) and mutagenesis ^{6, 8, 9}. Upon absorption, AFB₁ undergoes metabolic activation primarily by cytochrome P450 monooxygenases ^{12–14}, rendering an exceedingly reactive metabolite, *i.e.*, AFB₁-*exo*-8,9-epoxide ^{15–18}. The *exo*-epoxide interacts with DNA predominantly through binding to guanine residues at N7 position forming *trans*-8,9-dihydro-8-(N7-guanyl)-9-hydroxyafatoxin B₁ (AFB₁-N7-Gua) adducts, although small quantities of the corresponding adenine adducts can also be formed ^{15, 16, 19–21}. Alternatively, the highly reactive AFB₁-*exo*-8,9-epoxide can be detoxified through conjugation with glutathione catalyzed by glutathione *S*-transferase, or conversion to AFB₁-dihydrodiol catalyzed by microsomal epoxide hydrolase (*reviewed in* ^{8, 9, 18}). The predominant AFB₁-N7-Gua is a cationic labile adduct, which can either depurinate to yield abasic (AP) sites, or undergo a base-catalyzed rearrangement through which the imidazole ring of dG opens and a stable 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimidin-5-yl-formamido)-9-hydroxyafatoxin B₁ (AFB₁-FAPY) adduct is formed ^{8, 9, 16, 22}.

The unique features of *TP53* mutations in AFB₁-associated HCC provide an attractive context to investigate the underlying mechanism of carcinogenesis through correlation studies of AFB₁-DNA adduction and mutagenesis. Attempts have been made to recapitulate the characteristic *TP53* mutagenesis in human HCC by investigating the formation and kinetics of repair of AFB₁-induced DNA adducts or by establishing the AFB₁-induced mutagenesis in various *in vitro* and *in vivo* model systems (*reviewed in* ^{8, 9, 11, 18}). To this date, however, the correlation between base-specific AFB₁-induced DNA adducts and mutagenesis has not been investigated in a single mammalian model system. To simultaneously investigate AFB₁-induced DNA adduction and mutagenesis, we have used the transgenic Big Blue[®] mouse model system ²³, which is proven to be an invaluable means for correlation studies of DNA damage and mutagenesis at both genomic and single nucleotide level ^{24, 25}. Here, we have determined the induction of AFB₁-DNA adducts in the genome overall and specifically in the *cII* transgene of Big Blue mouse embryonic fibroblasts treated *in vitro* with AFB₁. We have also investigated the mutagenic fate of the induced AFB₁-DNA adducts by establishing the mutant frequency and mutation spectrum of the *cII* transgene in the AFB₁-treated cells. Subsequently, we have elicited correlations between AFB₁-DNA adduction and mutagenesis both at the genomic and single nucleotide levels. Methodologically, we have used an immuno-dot-blot assay with an AFB₁-DNA adduct-specific antibody ²⁶, and a lesion-specific cleavage assay with a specialized DNA repair enzyme ²⁷ to determine AFB₁-induced DNA adduction in the genome. We have utilized the terminal transferase-dependent polymerase chain reaction (TD-PCR) assay ²⁸ for footprinting of AFB₁-induced DNA adducts, at the nucleotide resolution level, in the *cII*

transgene. We have employed the λ Select-*cII* assay and DNA sequence analysis to establish AFB₁-induced *cII* mutant frequency and mutational spectrum, respectively.

Materials and Methods

Cell culture and chemical treatment

Early passage Big Blue mouse embryonic fibroblasts were grown as monolayers at ~20–25% confluence in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. Prior to chemical treatment, the culture media were removed, and the cells were washed thoroughly with phosphate buffered saline. The culture dishes were filled with serum free DMEM, and subsequently AFB₁ (Sigma-Aldrich Inc. Saint Louis, MO), dissolved in dimethylsulfoxide (DMSO), was added to the media at increasing concentrations of 0.04–100 μ M, in the presence and absence of standard S9-activation system^{29, 30}. The S9-activation system comprised of Aroclor 1254-induced rat liver preparations and cofactor reagents (Moltox, Inc. Boone, NC). The freshly made S9-activation system, prepared according to the manufacturer's instructions (Moltox, Inc.), was added to the media at a final concentration of 1%, and incubation was performed at 37°C for 6 hours in the dark. Immediately after treatment, the cells were harvested by trypsinization for evaluation of cytotoxicity and DNA damage formation. Alternatively, the treated cells were cultured in complete growth medium for an additional 4 days, and afterward were subjected to mutation analysis of the *cII* transgene. The 4-day growing period is essential for the fixation of all mutations into the genome²⁴. At the time of harvesting, all cultured cells had undergone 3–4 population doublings. All experiments were conducted in triplicates.

Immuno-dot-blot assay

To assess the formation of AFB₁-induced DNA adducts, genomic DNA of differently treated cells *vs* control were subjected to immuno-dot-blot assay using the mouse monoclonal anti-AFB₁ (6A10) antibody (Novus Biologicals, Inc. Littleton, CO), which is raised against the imidazole ring-opened persistent form of the major N7 guanine adduct of AFB₁ 26. The immuno-dot-blot assay was performed as described earlier³¹ with some modifications (*see*, "Supplementary Material").

Lesion-specific cleavage assay with AP endonuclease

To determine the formation of AFB₁-induced AP sites, genomic DNA of differently treated cells *vs* control were digested with AP endonuclease (APE), a multifunctional enzyme with DNA repair activity that cleaves DNA at AP sites²⁷ (*see*, "Supplementary Material").

TD-PCR assay

To map the formation of AFB₁-induced DNA adducts in the *cII* transgene, we have used the versatile TD-PCR footprinting assay, which enables sensitive and specific detection of DNA adducts at the level of nucleotide resolution^{28, 32} (*see*, "Supplementary Material").

cII mutant frequency and mutation spectrometry analyses

To establish AFB₁-induced *cII* mutant frequency and mutational spectrum, we have used the λ Select-*cII* assay (Stratagene, La Jolla, CA) and DNA sequence analysis, respectively (*see*, "Supplementary Material").

Results

Cytotoxicity examination

AFB₁ treatment of mouse embryonic fibroblasts, in the absence of S9-activation system, caused concentration-dependent cytotoxicity, most visibly in the micromolar dose range, as determined by the trypan blue dye exclusion assay (Fig. 2). In the presence of S9-activation system, however, AFB₁ treatment, in the corresponding dose range, resulted in much intensified cytotoxicity. In the latter case, cell survival was diminished to as low as $67.0 \pm 3.5\%$ and $16.5 \pm 6.7\%$ (Median $\pm$ SD) at doses of 5 and 10 μ M AFB₁, respectively. In keeping with the objective of our study to investigate AFB₁-induced DNA adducts in relation to mutagenesis, we determined a relevant dosing range to establish viable and proliferative cells in which the induced AFB₁-DNA adducts could be efficiently translated into mutations. Thus, we set the upper dose limit of AFB₁ for all DNA adduct and mutagenesis experiments no higher than 5 μ M, which rendered a moderate cytotoxicity, in the presence of S9-activation system, and a marginal cytotoxicity in the absence thereof (*see*, Fig. 2). For verification purposes, we also performed total cell counts in AFB₁-treated cells over defined periods of time post-treatment, and ensured that the proliferative capacity of the cells was not compromised subsequent to various AFB₁ treatments (*data not shown*).

DNA adducts in the genome

We used immuno-dot-assay and lesion-specific cleavage assay coupled to alkaline agarose gel electrophoresis to assess the formation of DNA adducts in AFB₁-treated mouse embryonic fibroblasts. Our immuno-dot-blot assay results verified a dose-dependent formation of persistent imidazole ring-opened (AFB₁-FAPY) adducts in the treated cells, in the presence of S9-activation system (*see*, Fig. 3a). In the absence of S9-activation system, however, there was no appreciable formation of AFB₁-FAPY adducts in the treated cells at all doses tested. To determine the presence of parent AFB₁-N7-Gua adducts in AFB₁-exposed cells, we pre-treated their genomic DNA with 15 mM Na₂CO₃, 30 mM NaHCO₃, pH 9.6 for 4 hours at 37°C. This pre-treatment is known to convert the cationic AFB₁-N7-Gua adducts to the imidazole ring-opened form³⁰. As shown in Figure 3b, there was no considerable difference in the detected levels of AFB₁-FAPY adducts between the pre-treated and non-pre-treated samples. This implies that the initially formed AFB₁-N7-Gua adducts were mainly converted to the persistent imidazole ring-opened derivatives by the end of AFB₁ treatment in cell culture. Moreover, our APE-cleavage assay in combination with gel electrophoresis analysis ruled out the formation of AP sites, at a detectable rate, in the genome of AFB₁-treated cells, in the presence or in the absence of S9-activation system (*see*, Fig. 3c). This can be inferred from the absence of detectable fragmentation of the genomic DNA in AFB₁-treated cells digested with APE, since the DNA repair function of this enzyme serves to cleave DNA at AP sites²⁷.

DNA adducts in the *cII* transgene

We used the TD-PCR assay to map the formation of DNA adducts, at the level of nucleotide resolution, in the *cII* transgene of AFB₁-treated mouse embryonic fibroblasts^{24, 28}. As shown in Figure 4, there was a dose-dependent and sequence-specific formation of AFB₁-DNA adducts in the treated cells, in the presence of S9-activation system. In the absence of S9-activation system, however, there was no significant formation of AFB₁-DNA adducts in the treated cells at all doses tested (*see*, Fig. 4). Preferential formation of AFB₁-DNA adducts along the *cII* transgene was at nucleotide positions: 7–11 (CGTG), 19 (C), 22–25 (AACG), 47–48 (CG), 62–64 (CG), 90–95 (GGAAGC), 101–105 (GCGTT), 120–135 (CAGCAGGTGGAAGAGG), 144–146 (TCC), 159–169 (GCTGCTTGCTG), 178–187 (TGGGGGGTTCG), 192 (G), 210–215 (GGCGCG), 225–228 (TGCG), and 247–256 (CGCCCGCG). All but one of these guanine-containing nucleotide positions (144–146 nt) encompass CpGs within their sequence contexts.

cII mutant frequency and mutation spectrum

We used the λ Select-*cII* assay to determine the induction of *cII* mutant frequency in AFB₁-treated mouse embryonic fibroblasts. As shown in Figure 5 and Table S1, AFB₁ treatment of these cells, in the presence of S9-activation system, resulted in a dose-dependent induction of *cII* mutant frequency. The induced *cII* mutant frequency reached a 4.1-fold-increase relative to background at the highest administered dose of AFB₁ ($P < 0.001$). In contrast, AFB₁ treatment of these cells, in the absence of S9-activation system, was not mutagenic at none of the tested doses as it only marginally elevated the *cII* mutant frequency relative to background (see, Fig. 5 and Table S1). The small increases in relative *cII* mutant frequency in cells treated with rising concentrations of AFB₁, in the absence of S9-activation system, were not appreciably different from one another, either.

To establish the spectra of AFB₁-induced and control mutations, we sequenced the DNA of *cII* mutants obtained from cells treated with 4 μ M AFB₁ or control solvent (DMSO), in the presence of S9-activation system. We randomly selected 95 *cII* mutant plaques induced by AFB₁ treatment in comparison to 100 control plaques. Of the respective number of plaques, 89 and 95 contained a minimum of one mutation in the *cII* transgene. As shown in Table S2, single mutations, mainly single base substitutions, predominated in both the AFB₁-induced and control mutation spectra. Detailed mutation spectra induced by AFB₁ treatment as compared with control are illustrated in Figure 6. The overall spectrum of mutations induced by AFB₁ treatment was significantly different from that of control ($P < 0.0001$).

To specify the difference between AFB₁-induced and control mutation spectra, we compared the frequency of each type of mutation, *e.g.*, transversions, transitions, etc. in the respective mutation spectra. In the Big Blue system, the *cII* transgene is a non-transcribed gene²⁵. Hence, the strand bias of mutagenesis, a phenomenon caused by transcription-coupled DNA repair³³, is unlikely to influence the spectrum of mutations found in this transgene. Conventionally, therefore, the strand mirror counterparts of all transversions (*e.g.*, G to T + C to A) or transitions (*e.g.*, G to A + C to T) are combined when the specific types of mutation between different treatment groups are compared²⁴. As shown in Table 1, G:C to T:A transversions prevailed in the spectrum of mutations produced by AFB₁ treatment, *i.e.*, 65.6% of all induced mutations *vs* 8.3% of control mutations ($P < 0.0001$). Approximately, half of these G:C to T:A transversion mutations induced by AFB₁ treatment occurred within CpG sequence contexts. For the most part, the CpG-targeted G:C to T:A transversions co-localized with preferential sites of AFB₁-DNA adduction along the *cII* transgene (see Fig. 4 and Fig. 6). However, not all hypermutable sites in the *cII* transgene in AFB₁-treated cells were necessarily the major hotspots of AFB₁-DNA adduction. For example, the frequently mutated codons 9, 30, and 60 in the *cII* transgene of AFB₁-exposed cells were not the strongest DNA-adduct formation sites. Conversely, the main hotspots of DNA adduction in the *cII* transgene of AFB₁-treated cells were not the most highly mutated positions. Of significance, codon 42 and codon 45 of the *cII* transgene, which have identical sequence contexts to that of human *TP53* codon 249, were two frequently mutated codons in AFB₁-treated cells, harboring the signature mutation of G to T transversion at their 3rd base positions^{7–11} (AGG to AGT) (see, Fig. 6).

Discussion

Epidemiologic studies have the potential to identify suspect carcinogens, which may etiologically be involved in human cancers³⁴. This objective is often achieved by the demonstration of a significant association between exposure to specific carcinogens and the incidence of cancer in defined human populations²⁴. Not only can the specificity of carcinogen exposure determine the type of human cancers, but it may also influence the genetic and/or epigenetic aberrations that are unique for certain malignancies²⁴. The correlative nature of human cancer development and carcinogen exposure can be used for causality inference at the

molecular level if the observed correlation can be recapitulated experimentally^{10, 11, 35}. Human HCC associated with dietary AFB₁ exposure represents an exceptional case for inferring cancer causality²⁴. The HCC tumor samples from individuals residing in areas with known contamination of foodstuffs by AFB₁ commonly harbor *TP53* mutations with distinctive mutational features, *i.e.*, G to T transversion at the 3rd base position of codon 249 (AGG to AGT), and a typical mutational spectrum predominated by G:C to T:A transversions^{7–11}. This mutation is exceedingly rare in cancers other than HCC and in HCC of patients from areas of the world where contamination of foodstuff with AFB₁ is low. To recapitulate these unique features of *TP53* mutations in AFB₁-associated HCC, we have investigated AFB₁-induced DNA adduction in relation to mutagenesis in transgenic mouse embryonic fibroblasts treated *in vitro* with AFB₁.

In keeping with the objective of our study to correlate AFB₁-induced DNA adducts and mutations at a physiologic level, we initially determined AFB₁ cytotoxicity to mouse embryonic fibroblasts. We^{29, 30} and others^{36, 37} had previously shown that *in vitro* AFB₁ requires an external metabolic activation system to exert its biological effects. We had also verified that the herein used rat liver S9-activation system, similarly to human liver S9 preparations or the classical double-induced S9 mix (Aroclor + phenobarbital or dexamethasone), has the capacity to convert AFB₁ to DNA reactive derivatives *in vitro*^{29, 30}. The rat liver S9-activation system is proven to contain cytochrome P450 3A4 and 1A2 enzymes³⁸ that are primarily involved in oxidative bioactivation of AFB₁^{12–14}. Thus, we included the S9-activation system in our cytotoxicity experiments and established a relevant dosing range for AFB₁, which served as a guideline for DNA adduct and mutagenesis experiments (*see*, Fig. 2).

Determination of DNA adducts in the genome overall and specifically in the *cII* transgene of AFB₁-treated mouse embryonic fibroblasts confirmed that S9-activation system is necessary for biotransformation of AFB₁ to DNA reactive species in this experimental model system (*see*, Fig. 3 and Fig. 4). Our immuno-dot-blot assay results revealed that AFB₁ treatment of these cells, in the presence of S9-activation system, gave rise to a dose-dependant formation of persistent imidazole ring-opened (AFB₁-FAPY) adducts in the genomic DNA (*see*, Fig. 3a). The parent AFB₁-N7-Gua adducts were not, however, detectable in the genome of AFB₁-treated cells, which implies an *in situ* rearrangement of these labile adducts to the stable AFB₁-FAPY adducts (*see*, Fig. 3b). Furthermore, our APE-cleavage assay coupled to gel electrophoresis analysis ruled out the formation of AP sites, at a significant rate, in the genomic DNA of AFB₁-treated cells (*see*, Fig. 3c). In the mammalian genome, AFB₁-FAPY adducts have been shown to evade DNA repair and persist for several days to weeks^{39, 40}. AFB₁-FAPY adducts have also displayed higher mutagenic potency and stronger blocking effects on DNA replication relative to their molecular predecessors (AFB₁-N7-Gua adducts)²². Nuclear magnetic resonance studies have demonstrated that AFB₁-FAPY adducts have a more subtle effect on DNA architecture and a pronounced effect on the melting temperature of the DNA duplex than AFB₁-N7-Gua adducts^{41, 42}. These properties enable the AFB₁-FAPY adducts to evade DNA repair and exhibit great mutagenic potentials^{40, 43, 44}.

In confirmation, our TD-PCR footprinting data verified a dose-dependent and sequence-specific formation of DNA adducts in the *cII* transgene of AFB₁-treated cells, in the presence of S9-activation system only (*see*, Fig. 4). Preferential formation of AFB₁-induced DNA adducts along the *cII* transgene was almost exclusively localized to guanine-containing sequences encompassing CpG dinucleotides. The high reactivity of AFB₁ with guanine residues, and its sequence context selectivity for guanines adjacent to cytosines on the 5' end, have been demonstrated both *in vitro* and *in vivo*^{9, 11, 14, 20, 22, 36, 45, 46}. Of relevance, we have previously shown that all CpGs in the *cII* transgene of the Big Blue mouse system are highly methylated⁴⁷.

Consistent with the above-mentioned AFB₁-DNA adduction data, our mutation analysis of the *cII* transgene in AFB₁- treated mouse embryonic fibroblasts showed a significant and characteristic mutagenic response in the treated cells, in the presence of S9-activation system only. More specifically, there was a dose-dependent induction of *cII* mutant frequency in AFB₁- treated cells, which reached a 4.1-fold-increase relative to background at the highest administered dose of AFB₁ ($P < 0.001$) (see, Fig. 5 and Table S1). The AFB₁- induced *cII* mutation spectrum differed significantly from that of control ($P < 0.0001$), and was characterized by a predominant induction of G:C to T:A transversions occurring mostly within CpG sequence contexts (see, Fig. 6 and Table 1). Notably, codon 42 and codon 45 of the *cII* transgene, which have the same sequence contexts as codon 249 of human *TP53* gene, constituted two frequently mutated sites in AFB₁-treated cells, harboring the signature mutation of G to T transversion at their 3rd base positions (see, Fig. 6). These findings are in good agreement with previously reported results by others showing that sequence-specific G:C to T:A transversions are the hallmark of mutations produced by AFB₁ in various *in vitro* or *in vivo* model systems^{14, 20, 22, 36, 45, 46}. Aguilar *et al.*⁴⁸ have shown a preferential induction of G to T transversions at the 3rd base position of codon 249 in AFB₁-treated human hepatocarcinoma cells (HepG2), although other types of mutations in neighboring bases were also observed, albeit at much lower frequencies. For example, the absolute frequencies of AFB₁-induced G to T transversions at the 2nd and 3rd base positions of codon 249 were 0.8 and 8.4×10^{-7} , respectively. Methodologically, the occurrence of the former types of mutations could partially be ascribed to a misincorporation of DNA polymerase during the amplification step of the restriction fragment length polymorphism-PCR used in the Aguilar *et al.*⁴⁸ study. In fact, the relative frequencies of G to T transversions at the 2nd base position of codon 249 in AFB₁-treated cells and control were 4.3% and 4.5%, respectively⁴⁸.

Moreover, there was a good concordance between our DNA footprinting and mutagenicity data as the CpG-targeted G:C to T:A transversions in the *cII* transgene accorded well with the preferential formation of AFB₁- DNA adducts found therein (see, Fig. 4 and Fig. 6). This implies that AFB₁- DNA adduction essentially drives the induction of mutagenesis in our experimental model system. However, not all sites of AFB₁- DNA adduction in the *cII* transgene were hypermutable *per se*. For example, whereas the CG rich nucleotide positions 247–256 were a major hotspot of AFB₁-DNA adduction, no mutation was detectable in these sequences. Of importance, mutations in the last 1/3rd part of the *cII* gene does not severely impair the function of the cII protein²³. Thus, although AFB₁-DNA adduction is a driving force behind AFB₁-induced mutagenesis in our experimental system, it cannot, in and of itself, elicit the mutagenic outcome. It appears that the formation of AFB₁-DNA adducts in critically important genomic loci is integral for a sequential chain of events, which may act additively or synergistically in relation to one another.

The extent to which AFB₁-DNA adduction, in addition to or in combination with other determining factors, *e.g.*, viral infection, drives its mutagenicity and carcinogenicity is an important area of research, which requires further exploration^{9, 11}. We have previously demonstrated that while AFB₁-DNA adducts were substantially formed in the 2nd guanine of codon 249 of the *TP53* gene in human liver cells, more extensive formation of these adducts was also detectable in other positions in several additional codons³⁰. This situation is well reflected in the present study as the hypermutable codon 42 and codon 45 of the *cII* transgene were two substantial, but by no means the sole or the most significant, AFB₁-DNA adduct formation sites. As explained above, the major hotspots of AFB₁-DNA adduction in the *cII* transgene were not necessarily the most frequently mutated positions, either. Thus, these observations suggest that additional biological mechanism may contribute to the predominance of G to T transversions in the 2nd guanine of codon 249 of the *TP53* gene in human HCC.

Lastly, we should acknowledge that in the present study, we have used mouse embryonic fibroblasts as a surrogate for human hepatocytes, which are the target cell type for AFB₁-mutagenicity. Species- or cell type-dependent influential factors that contribute to mutagenesis, such as metabolic activity, DNA repair capacity, fidelity of polymerases for DNA synthesis, etc.²⁴ should be taken into account while interpreting the data obtained in model systems, and finding their relevance to humans.

In conclusion, we have shown a DNA adduct-mediated mutagenicity of AFB₁ in transgenic mouse embryonic fibroblasts, both at the genomic and single nucleotide levels. Although the correlated aspects of AFB₁-DNA adduction and mutagenesis in this experimental system recapitulate the unique mutational features of the *TP53* gene in AFB₁-associated human HCC^{1, 7}, they also reiterate the multifactorial nature of the etiology of this disease^{4, 5}. Notwithstanding the crucial role of AFB₁-DNA adducts in inducing mutagenesis, our findings support an interplay among different etiologic factors in shaping the signature of *TP53* mutations in dietary AFB₁-associated human HCC. Altogether, DNA adduct-derived mutagenicity of AFB₁, in addition to or in combination with other determinants, is likely to account for the mutation signature of this tumor type.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AP	abasic site
APE	AP endonuclease
AFB₁-FAPY	8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxyafatoxin B ₁
AFB₁-N7-Gua	<i>trans</i> -8,9-dihydro-8-(N7-guanyl)-9-hydroxyafatoxin B ₁
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
HCC	hepatocellular carcinoma
LM-PCR	ligation-mediated polymerase chain reaction

TD-PCR

terminal transferase-dependent polymerase chain reaction

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Figure 1. *TP53* gene mutations in hepatocellular carcinoma

(a) Codon distribution and (b) mutation spectrum of the *TP53* gene in all HCC cases ($n = 693$). (c) Codon distribution and (d) mutation spectrum of the *TP53* gene in HCC cases with known exposure to AFB₁ ($n = 53$). Data were obtained from the IARC *TP53* mutation database (R13, November 2008)¹⁰. Note that 81% of all HCC cases reported in the database originate from regions where components of staple diets are often moderately or highly contaminated with AFB₁. Del = Deletions; Ins = Insertions.

Figure 2. Cytotoxicity of AFB₁ to Big Blue mouse embryonic fibroblasts

Cell cultures were treated with increasing concentrations of AFB₁ or control solvent (DMSO), in the presence and absence of standard S9-activation system^{29, 30} (*see*, Materials and Methods). Subsequently, cell viability was determined by the trypan blue dye exclusion assay. Viability is expressed as a percentage of total cell number. Error bars = SD.

Figure 3. Determination of AFB₁-induced DNA damage in the mouse genome

Mouse embryonic fibroblasts were treated with increasing concentrations of AFB₁ or control solvent (DMSO), in the presence and absence of standard S9-activation system^{29, 30} (see, Materials and Methods). **(a)** Genomic DNA was isolated and subjected to immune-dot-blot assay using the mouse monoclonal anti-AFB₁ (6A10) antibody²⁶. **(b)** To determine the presence of parent AFB₁-N7-Gua adducts, genomic DNA was alkali-pre-treated to convert the cationic AFB₁-N7-Gua adducts to the imidazole ring-opened form (AFB₁-FAPY)³⁰. (+) and (-) conversion indicate the genomic DNA pre-treated and non-pre-treated, respectively. **(c)** To determine the formation of AP sites, genomic DNA was digested with AP endonuclease (APE) and the purified DNA digests were subjected to alkaline agarose gel electrophoresis, followed

by standard visualization procedure. (+) and (–) represent the presence and absence, respectively, of the APE enzyme in the reaction mix.

M = molecular size marker; Pos. Ctrl = Positive Control.

Figure 4. Mapping of AFB₁-induced DNA adducts in the *cII* transgene

TD-PCR footprinting of the full-length *cII* transgene was done using the genomic DNA of mouse embryonic fibroblasts treated with increasing concentrations of AFB₁ or control solvent (DMSO), in the presence and absence of standard S9-activation system^{29, 30} (*see*, Materials and Methods). In addition to the IRDye[®] 700 Sizing Standards (LI-COR), Maxam and Gilbert chemical reactions⁴⁹, prepared from control genomic DNA and subjected to ligation-mediated PCR (LM-PCR)³², were run in parallel to all samples. The LM-PCR bands migrate approximately 3 bases faster than the corresponding TD-PCR bands due to the addition of three riboguanosine triphosphate to all primer extension products in the latter method^{24, 28}. bp = base pair; M = molecular size marker; nt = nucleotide position.

Figure 5. AFB₁-induced and control *cII* mutant frequency data

Mutant frequency of the *cII* transgene in Big Blue mouse embryonic fibroblasts treated with increasing concentrations of AFB₁ or control solvent (DMSO), in the presence and absence of standard S9-activation system^{29, 30} (see, Materials and Methods).

Figure 6. AFB₁-induced and control *cII* mutation spectrometry data

Detailed mutation spectra of the *cII* transgene in mouse embryonic fibroblasts treated with 4 μ M AFB₁ or control solvent (DMSO), in the presence of standard S9-activation system^{29, 30} (see, Materials and Methods). The AFB₁-induced mutations are typed in capital letters above the reference *cII* sequence, whereas the control mutations are typed in small letters below the reference *cII* sequence. Deleted bases are underlined. Inserted bases are shown with an arrow. Numbers below the bases are the nucleotide positions.

Table 1 Comparative mutation spectra of the *cH* transgene in Big Blue mouse embryonic fibroblasts treated with AFB₁ or control

Mutation type	Number of Mutations		% Mutations		Absolute Mutant Frequency ($\times 10^{-5}$)	
	AFB ₁	Control	AFB ₁	Control	AFB ₁	Control
G:C→C:G	7	5	7.8	5.2	1.55	0.25
G:C→T:A (at CpG)*	59 (29)	8 (1)	65.6 (32.2)	8.3 (1.0)	13.00 (6.38)	0.40 (0.05)
G:C→A:T	13	42	14.4	43.8	2.85	2.11
A:T→T:A	2	4	2.2	4.2	0.44	0.20
A:T→G:C	0	13	0	13.5	0	0.65
A:T→C:G	5	14	5.6	14.6	1.11	0.70
Del/Ins	4	10	4.4	10.4	0.87	0.50

* The number of G:C→T:A transversion mutations occurring specifically at CpG dinucleotides is indicated into brackets.
Del = deletion; Ins = insertion.