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AUTHOR'S VIEW

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Early detection of the aflatoxin B_1 mutational fingerprint: A diagnostic tool for liver cancer

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ABSTRACT

Using duplex-consensus sequencing technology, we recently identified the characteristic high-resolution mutational spectrum of the liver carcinogen aflatoxin B_1 in a mouse model, many months before aflatoxin-induced tumors are detectable. The diagnostic power of this spectrum is then demonstrated by accurately identifying, among the sequenced human liver tumors, the subset of cancers associated with aflatoxin B_1 exposure.

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The lethality of cancer is due, in part, to late detection. Among the worst offenders is hepatocellular carcinoma (HCC), a common form of liver cancer that progresses relatively asymptomatically to advanced stages for which there are very limited therapeutic options. Among the many etiological factors for HCC, fungal-toxin exposure and chronic viral hepatitis are the most prominent,[1] especially in combination.[2] These factors are endemic to the developing world such as sub-Saharan Africa, Central and South America, and Southeast Asia, where inadequate food storage practices often lead to fungal contamination of staple crops.[3] Given its prevalence in high-risk areas, HCC is the second leading cause of cancer death worldwide, with over 740,000 deaths recorded in 2012.[4] Given these considerations, there is a substantial medical need for early detection of HCC, and more specifically, for early detection of the mutagenic processes that underlie genesis of the disease.

Our recent work tackled this problem by identifying the early mutational events that accompany exposure to the fungal toxin and potent liver carcinogen aflatoxin B_1 (AFB_1).[5] We used the B6C3F1 (the F1 progeny from a female C57BL/6J mouse with a male C3H/HeJ mouse) mouse model of liver cancer in which a single dose of AFB_1, given in infancy, induces tumors in 100% of the male animals, one year and a half later.[6] However, for the first few months (e.g., at 10 weeks post treatment), the treated livers are indistinguishable from controls, even though they are irreversibly committed to developing cancer. This feature of the mouse model recapitulates well human HCC.

As current conventional sequencing technologies lack the fidelity of finding rare, "needle in the haystack" mutations in a largely normal tissue, we developed an ultra-high-fidelity sequencing protocol by combining the highly accurate duplex-consensus sequencing method developed by our collaborator[7] with the genetic features of the transgenic mouse model, *λ-gptΔ* B6C3F1. This variant of the B6C3F1 mouse features 40 repeats of a λ-phage vector harboring a 6.4-kb insert on chromosome 17[8]; these viral cassettes can be isolated via a phage packaging reaction, and amplified in Cre-expressing bacteria to recover, via Cre-Lox recombination, the 6.4-kb insert as a plasmid.[8] (As indicated in the name of the mouse strain, the plasmid also contains a copy of the bacterial guanine phosphoribosyltransferase (*gpt*) gene, but its presence is irrelevant for our sequencing analysis.) By deep sequencing the 6.4-kb target plasmid, mutational processes previously operating or still ongoing in the mouse liver can be identified by their characteristic mutational spectra.[5] Analysis of DNA sequences was performed using the duplex-consensus error-correction strategy, in which DNA strands are individually barcoded before sequencing, and true mutations are called only in the event that they are present in both of the complementary strands of a given DNA molecule.[7] This error-correction process provided 3–4 orders of magnitude higher fidelity than conventional next-generation sequencing which, combined with a high sequencing depth (average depth of coverage per base ~15,000), allowed us to find the rare, carcinogen-induced mutations likely responsible for liver cancer initiation.[5]

The animal experiment and results are detailed in Fig. 1. Four-day-old B6C3F1 male mice were given a 6 mg/kg dose of AFB_1 dissolved in dimethyl sulfoxide (DMSO); control animals received DMSO only. At 10 weeks and 72 weeks post exposure, animals were killed and their livers isolated. At 10 weeks, the livers of treated animals look indistinguishable from the untreated

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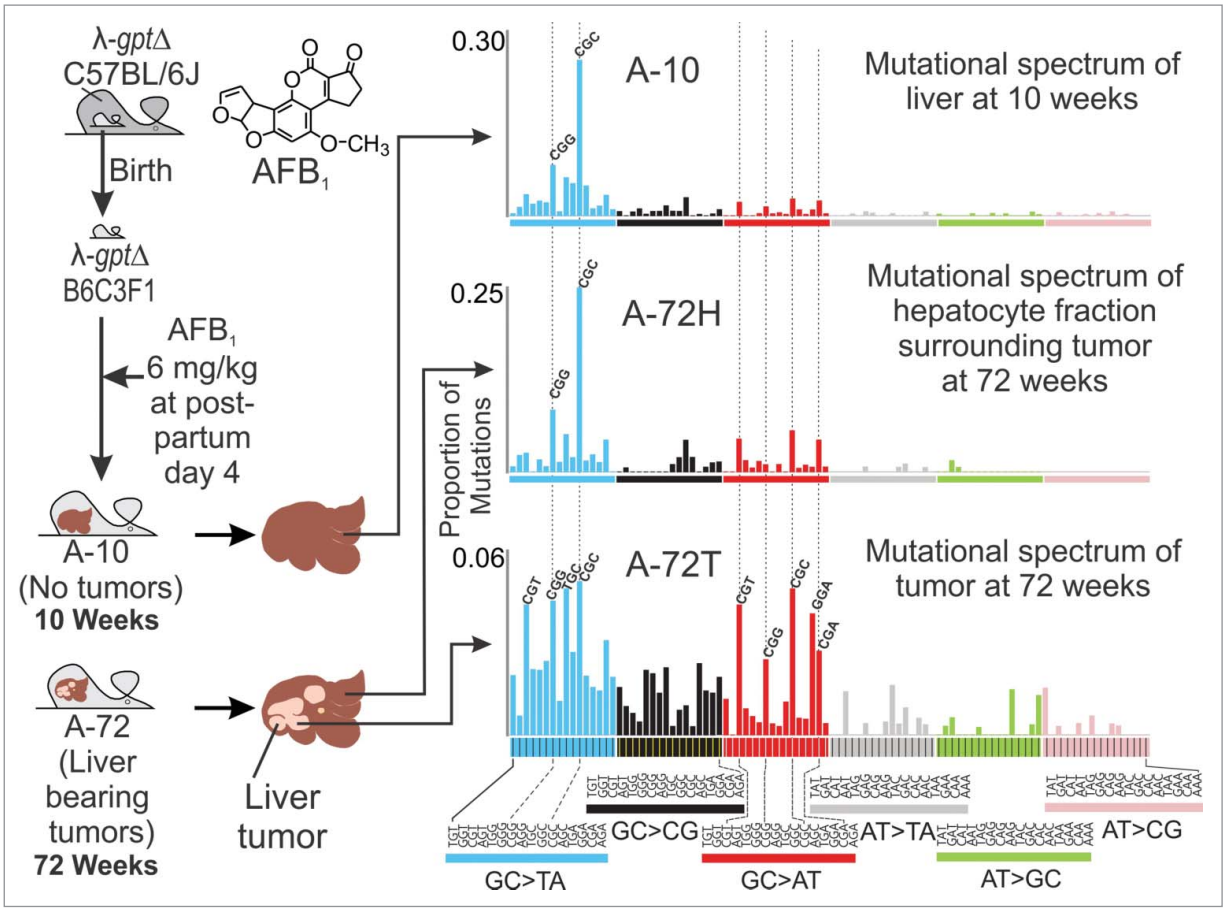

Figure 1. Recording the mutational spectra of aflatoxin B_1 (AFB_1) in a mouse model. Four-day-old *λ-gptΔ* B6C3F1 mice (resulting from the cross of a female *λ-gptΔ* C57BL/6J mouse with a male C3H/HeJ mouse) were administered a dose of 6 mg/kg of AFB_1. At 10 weeks and 72 weeks post treatment, mice were killed and their livers were isolated. Genomic DNA was extracted from 10-week-old bulk liver, 72-week-old liver tumor and 72-week-old hepatocyte fraction surrounding the tumor. Using a high-resolution sequencing technology,[5] the mutational spectra induced by AFB_1 were determined. Samples and spectra associated with 10-week-old animals are denoted as A-10; samples and spectra associated with 72-week-old animals are denoted A-72; the tumor spectrum is labeled A-72T, while the spectrum of hepatocytes surrounding the tumor, A-72H. Each bar graph shows the proportion of point mutations (represented by the different colors) in each of the 16 possible trinucleotide sequence contexts. Certain dominant sequence contexts are highlighted.

controls. At 72 weeks, however, all treated animals have developed tumors. Genomic DNA was extracted from: (1) bulk liver at 10 weeks; (2) tumors at 72 weeks; and (3) the collagenase-sensitive cellular fraction (largely hepatocytes) surrounding the tumors at 72 weeks, and analyzed as outlined above. In each sample, the point mutations were enumerated and sorted by the triplet nucleotide sequence context in which they occurred. There are 6 possible types of point mutations; each can occur in 16 different trinucleotide sequence contexts, thus yielding a mutational spectrum of $6 \times 16 = 96$ possible 3-base context point mutations (Fig. 1).

Our data indicated that AFB_1 induced a unique, characteristic mutational spectrum, detectable as early as 10 weeks post exposure.[5] The AFB_1 spectrum at 10 weeks, denoted A-10, is dominated by GC→TA mutations, well known in the literature as being caused by the N7-guanine DNA adducts generated by AFB_1.[9] Strikingly, the distribution of these GC→TA mutations across the different sequence contexts was very uneven; 25% of mutations occurred in the CGC sequence context (Fig. 1). This is the first time that the CGC→CTC point mutation has been uniquely associated with AFB_1 exposure in an experimental animal model. By contrast, the DMSO control spectrum featured mutations more evenly distributed across G→T, G→C, and G→A point mutations.[5] At 72 weeks, the hepatocyte fraction spectrum (A-72H) was nearly identical to A-10, recapitulating its distinctive CGC hotspot (Fig. 1). The tumor spectra (A-72T), however, were more complex, suggesting that, while the contribution of the AFB_1-induced A-10 spectrum was still evident, additional mutagenic processes have been operational (Fig. 1).

As an early exposure spectrum, the mouse A-10 spectrum captures the intrinsic mutational characteristics of AFB_1 mutagenesis and, thus, may constitute a sensitive biomarker of exposure. To demonstrate its usefulness for detecting human aflatoxin exposure, we analyzed the mutational data from 314 sequenced human liver tumors.[5] An unsupervised hierarchical clustering of the human mutational spectra revealed a subset of 13 samples highly similar to A-10.[5] A number of these 13 liver tumors were previously predicted, based on epidemiological metadata, to come from AFB_1-exposed patients.[10] Therefore, our data provide tangible, experimental evidence for these previous correlations and showcase the diagnostic potential of mutational spectra.

Taken as a whole, our results suggest that the AFB_1 early exposure mutational spectrum we have identified can serve as a powerful biomarker, an early warning system that can inform

risk of liver cancer many years before tumors are apparent. Moreover, our approach can be generalized for other chemical carcinogens and expand the list of available mutational spectra biomarkers. Furthermore, the experimental platform described in our study[5] constitutes a versatile system to study not only cancer etiology and early detection, but also strategies for prevention, early intervention and potentially therapy.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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