

SHORT COMMUNICATION

Elevated *HPRT* mutation frequencies in aflatoxin-exposed residents of Daxin, Qidong County, People's Republic of China

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Molecular biomarkers are becoming increasingly important tools to identify people who are at highest risk of developing cancer. For many years we have been studying residents of Qidong County, People's Republic of China, to examine the combined impact of aflatoxin exposure with other risk factors as contributors to the high liver cancer incidence rates in this region. This study was conducted to determine the effects of aflatoxin exposure, as measured by serum aflatoxin-albumin adduct levels, on somatic mutation frequency in the human hypoxanthine guanine phosphoribosyl transferase gene (*HPRT*). Subjects were assigned as low or high according to a dichotomization around the population mean of aflatoxin-albumin adducts. *HPRT* mutant frequency was determined in individuals by a T cell clonal assay and the samples were categorized as low or high according to mean values. Separate analyses were also conducted for the small set of hepatitis B virus surface antigen (HBsAg)-positive and the larger set of HBsAg-negative individuals, known risk factors for liver cancer. An odds ratio of 19.3 (95% confidence interval 2.0, 183) was demonstrated for a high *HPRT* mutation frequency in individuals with high aflatoxin exposure compared with those with low aflatoxin exposure. This association indicates that aflatoxin-induced DNA damage in T lymphocytes, assessed using the validated surrogate albumin adduct markers, leads to increased mutations reflected as elevated *HPRT* gene mutations. This cross-sectional study suggests the potential use of mutation frequency of the *HPRT* gene as a long-term biomarker of aflatoxin exposure in high risk populations.

Aflatoxin B1 (AFB1) is known to be a major risk factor for the development of hepatocellular carcinoma (HCC) in many areas of the world (1–11). AFB1 exposure can be assessed by measuring AFB1 metabolite levels in urine or blood proteins and these biomarker measurements have been very valuable in population-based studies to determine this agent's role in the etiology of HCC (12–17). However, these adduct measurements will probably have a limited role in individual risk assessment unless they are paired with other longer term exposure and effect biomarkers. An example of a biomarker reflecting long-term exposure to DNA damaging agents are the somatic mutations that occur in the human X-linked hypoxanthine guanine phosphoribosyl transferase gene (*HPRT*) (18–26). The use of *HPRT* as a reporter gene for AFB1 exposure has not yet been defined in a human population, however, since mutation frequencies in human T cells can remain elevated for years after the specific insult (19,20,27), cumulative exposure to AFB1 could potentially be measured. Further, the *HPRT* mutant T lymphocyte assays are well-developed methods for detecting *in vivo* somatic cell gene mutations in humans (26,28–30).

AFB1 is a very potent mutagen and the *in vitro* mutational spectrum of AFB1 in exon 3 of the human *HPRT* gene in B lymphoblasts has been characterized. The mutational hotspot induced by AFB1 *in vitro* in exon 3 of the native human *HPRT* gene was a GC→TA transversion at base 209, occurring in 17% of AFB1-induced mutants (23). This information provided a basis to study *HPRT* mutation frequency in a high AFB1 exposure area in Qidong County, People's Republic of China (31), and we report for the first time a relationship between *HPRT* mutation and AFB1 exposure.

The study was conducted in Daxin Township, a community of ~40 000 residents in Qidong County, where HCC causes 10% of all adult deaths (31,32). The subjects were a healthy sub-group of individuals screened for participation in a chemoprevention trial with oltipraz that has been described previously (33,34). None of the study subjects in this investigation were active participants in the intervention trial but they had been deemed eligible for inclusion by an extensive physical and medical examination. The overall study cohort comprised 90 Chinese men and women aged 40–65 of whom 42 were male and 48 were female. Ten milliliter blood samples were drawn from the 90 participants into Leukoprep tubes. These samples were processed immediately to separate serum, red blood cells and white blood cells. They were sent within 48 h from the time of collection on ice to the USA for analysis of *HPRT* mutations. An additional 3 ml blood sample was drawn from the individuals for AFB1 analysis. In total, information on age, gender, hepatitis B virus surface antigen (HBsAg) status, leukocytes, serum glutamic pyruvic transaminase (SGPT), white blood cell count, hemoglobin and platelet counts were obtained. All of the samples were coded and each person who analyzed a sample was blind to the identity of the subject.

Abbreviations: AFB1, aflatoxin B1; HBsAg, hepatitis B virus surface antigen; HCC, hepatocellular carcinoma; *HPRT*, hypoxanthine guanine phosphoribosyl transferase gene; PHA, phytohemagglutinin; SGPT, serum glutamic pyruvic transaminase; 6-TG, 6-thioguanine.

Table I. Distribution of variables from the cohort in Qidong County, People's Republic of China

| Variable | Mutation frequency (no. of mutants per cell) | | | | | | OR | 95% CI |
|------------------------------------------|----------------------------------------------|---------|-----------------|--------------------------------------|---------|-----------------|--------------------|------------|
| | $\geq 26.3 \times 10^{-6}$ ($n = 11$) | | | $< 26.3 \times 10^{-6}$ ($n = 23$) | | | | |
| | No. | Percent | Mean \pm SE | No. | Percent | Mean \pm SE | | |
| Age (years) | | | 53 \pm 8 | | | 49 \pm 7 | | |
| Gender | | | | | | | | |
| Female | 3 | 27 | | 9 | 39 | | 1.00 | |
| Male | 8 | 73 | | 14 | 61 | | 0.58 | 0.12, 2.8 |
| Aflatoxin (pmol AFB1/mg albumin) | | | 0.70 \pm 0.12 | | | 0.41 \pm 0.19 | | |
| Low | 1 | 10 | | 15 | 68 | | 1.00 | |
| High | 9 | 90 | | 7 | 32 | | 19.30 ^a | 2.0, 183 |
| HBsAg | | | | | | | | |
| Negative | 10 | 91 | | 21 | 91 | | 1.00 | |
| Positive | 1 | 9 | | 2 | 9 | | 1.05 | 0.08, 12.9 |
| Leukocytes (urinalysis) | | | | | | | | |
| Negative | 10 | 91 | | 21 | 91 | | 1.00 | |
| Positive | 1 | 9 | | 2 | 9 | | 1.05 | 0.08, 12.9 |
| Clonal efficiency (%) | | | 2.0 \pm 1.0 | | | 6.0 \pm 5.0 | | |
| Clinical pathology results | | | | | | | | |
| SGPT (IU) | | | 19.7 \pm 4.7 | | | 18.0 \pm 5.3 | 1.07 | 0.92, 1.25 |
| White blood cells (per mm ³) | | | 6000 \pm 1468 | | | 5313 \pm 1154 | 1.00 | 0.99, 1.00 |
| Hemoglobin (g/dl) | | | 141 \pm 13 | | | 139 \pm 17 | 1.00 | 0.96, 1.06 |
| Platelets (thousands/mm ³) | | | 100 \pm 22 | | | 113 \pm 37 | 0.99 | 0.96, 1.01 |

^a $P < 0.05$.

After receipt of the samples for assay for *HPRT* mutation, the tubes were centrifuged again and the mononuclear cell fraction was washed twice in medium (RPMI 1640) by sedimentation, resuspended and frozen as described previously (29). For the cell cloning assay, the ampoules were thawed rapidly and the cell suspension added dropwise to 20 ml of RPMI 1640 containing 20% newborn bovine serum. After two washes by centrifugation, the cells were resuspended, counted, then diluted to 1×10^6 cells/ml and incubated for 36–40 h with 1 μ g/ml phytohemagglutinin (PHA). Following centrifugation and washing the cells were inoculated at 1, 2, 5 or 10 cells/well (non-selection) and 1 or 2×10^4 cells/well (selection) into microtiter plates. Growth medium consisted of RPMI 1640 medium containing 5% defined supplemented bovine calf serum (Sterile Systems, Grand Rapids, MI), 20% HL-1 medium (Ventrex, Ventura, CA), 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 10 μ g/ml streptomycin sulfate and optimal amounts of T cell growth factor and 0.125 μ g/ml PHA. Selection wells contained 10^{-5} M 6-thioguanine (6-TG). Wells also contained 1×10^4 irradiated (8000 rad) TK6 lymphoblastoid feeder cells, all in a total volume of 0.2 ml. Cells were incubated for 10–14 days and scored for colony growth by use of an inverted phase microscope.

Cloning efficiencies (non-selection and 6-TG selection) were calculated by the Poisson relationship $P_0 = e^{-x}$, which defines the average number of clonable cells/well (x), and factored by the number of cells added to wells. The cloning efficiency in selection divided by the non-selected cloning efficiency defines the measured mutant frequency reported as the frequency of 6-TG-resistant cells per 1 000 000 cells analyzed. Clones were also expanded for molecular analysis in modified RPMI 1640 containing feeder cells (2.5×10^5 cells/cm²) in increasingly larger size wells.

The human serum samples were assayed for the detection of AFB1–albumin adducts by methods that have been described in detail (31). Briefly, the samples were first concentrated by high speed centrifugal filtration using Microcon-50 micro-

concentrators (Amicon, Beverly, MA). The amount of human serum albumin resulting from this process was determined in each sample by a bromocresol purple dye binding method. Total serum proteins were then digested with Pronase prior to the measurement of AFB1 content by a radioimmunoassay. All statistical analyses were done using the statistical package STATA.

Of the 90 individuals for whom blood was collected, three of the 42 males and six of the 48 females were HBsAg seropositive. The ages of the cohort ranged from 40 to 65 years of age and the average age (\pm SD) was 50.9 (\pm 7.3) years for the males and 49.6 (\pm 6.3) years for the females. The average AFB1 measurement was 0.66 pmol AFB1/mg albumin and ranged from 0.27 to 1.19 pmol AFB1/mg albumin. Tests of hematology and blood chemistry, urinalysis and a liver function test gave no indications of hematological, renal or liver disease. The clonal efficiencies were extremely low for the *HPRT* T lymphocyte cloning assay (4.0%). Due to difficulty in the T cell cloning assay, many of the mutant frequencies (mutant frequency per clonable T cell) were not able to be determined, significantly reducing the sample size. Despite the smaller sample size of 34, the data maintain their robustness and statistical validity when variables known to be unassociated with mutant frequency are examined. The odds ratios for liver function (SGPT), white blood cells, hemoglobin and platelets with mutant frequency are consistent with tight confidence intervals (Table I).

Prior research has indicated the need to adjust *HPRT* mutant frequency values for age and cloning efficiency (29,35–37) and the values shown have been adjusted. Mutant frequencies were dichotomized for subsequent analysis based on the mean of the entire population (26.3×10^{-6} mutants/T cell) (Table I). Neither age nor gender was associated with *HPRT* mutant frequency.

AFB1 was dichotomized based on the mean of the population (0.66 pmol AFB1/mg albumin). Previous studies for AFB1 have dichotomized exposure based on the mean of the popula-

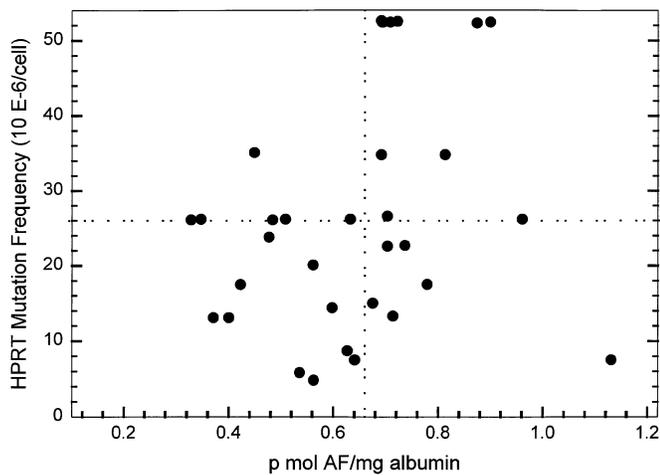


Fig. 1. The individual data values for aflatoxin albumin adducts (pmol AFB1/mg albumin) and *HPRT* mutation frequency are depicted. The vertical and horizontal dotted lines represent the mean values for each of these two biomarkers.

tion studied; the same has been done for this population. Since everyone in this population is exposed to some degree, dichotomization allows stratification of this exposed population by those who have very high levels of exposure and those who have moderate to low exposures to AFB1. For those distinguished as low *HPRT* mutant frequencies, the mean AFB1 was 0.41 ± 0.19 pmol AFB1/mg albumin. The mean AFB1 value for those with high mutant frequencies was 0.70 ± 0.12 pmol AFB1/mg albumin. In Figure 1, the relation between AFB1–albumin adduct levels and *HPRT* mutation frequency for the individual samples are shown by the dotted lines representing the mean values for these measures. An association between AFB1 and *HPRT* mutant frequency was demonstrated with an odds ratio of 19.3 (95% confidence intervals 2.0, 183) for high mutant frequency in those with a high exposure to AFB1, compared with those with a low exposure to AFB1.

This high risk population is exposed to a wide range of AFB1 levels (31) and it is suspected that only individuals with high levels of AFB1 exposure will develop DNA damage severe enough to be measured through reporter genes such as the *HPRT* gene. The use of this study cohort increases the likelihood of identifying an association between AFB1 and DNA mutations. That this cohort is also a high risk group for HCC (32,38,39) increases the possibility of detecting intermediate outcomes in the carcinogenic process such as mutations.

The typical mutant frequency for normal healthy adults in the *HPRT* gene is $5\text{--}8 \times 10^{-6}$ mutants/cell (22,23). The mean mutation frequency for this population is 26.3×10^{-6} mutants/cell. The significantly high mutant frequency indicates that this population suffers from exposure to environmental agents that lead to DNA damage. In this study, mutant frequencies of only 34 of 90 individuals were able to be analyzed for all markers. Thirty-six of the individuals had T cells which were not viable and could not be stimulated. An additional 20 individuals had T cells that were viable, but subsequent mutants were not able to be cloned. It is possible that shipping conditions and time taken to deliver the samples from China to Vermont may have affected the ultimate viability of the T cells. Equally likely, however, is that the individuals' blood samples reflect that of a population exposed to damaging environmental exposures that lead to appropriate external or

internal stimuli causing the cells to undergo cell death or apoptosis (40,41).

If the T cell viability was affected by the shipping conditions, then a selection bias on the remaining samples is not likely to have occurred. If T cell viability was due to heavily damaged cells, then the results would have been biased to one, as results from those with the most environmental damage would not have been obtained. For the remaining 34 samples, the range of mutant frequencies were consistent. Despite the difficulty in obtaining *HPRT* mutant frequencies and the final limited sample size, we believe that the mutant frequency values that were obtained are accurate and unbiased.

A major assumption in this study is that an individual's AFB1 measurement will be representative of the AFB1 exposure throughout their lives, even though AFB1–albumin adducts indicate recent exposure. Fortunately, the AFB1 measurements calculated in the study are comparable with the AFB1 measurements of previous year-long studies using this population (31). Further, the differences between the dichotomized AFB1 levels of low and high in this study are also consistent with that of previous studies.

Measurement of AFB1–albumin adducts is a valid method to reliably and sensitively measure AFB1 exposure. Similarly, the T lymphocyte assay is the most reliable somatic mutation assay for measuring *HPRT* mutant frequencies. Furthermore, our population is well studied and their high exposures to AFB1 lends itself to studying subsequent effects. The significantly positive odds ratio of 19.3 associating high AFB1–albumin adducts with high *HPRT* mutant frequencies is therefore consistent with the hypothesis that *HPRT* mutant frequencies are a reflection of AFB1-induced DNA damage.

A limitation to this study is the cross-sectional design. By definition, a cause and effect relationship cannot be ascertained from a cross-sectional study; it is unknown whether the exposure leads to the outcome or whether the outcome preceded the hypothesized exposure. In this case, however, biological plausibility indicates that AFB1 exposure leads to AFB1–albumin and AFB1–DNA adducts, which in turn lead to DNA damage severe enough to be reported as *HPRT* gene mutations.

The association between AFB1 and *HPRT* mutant frequency might be diluted due to exposure/AFB1 misclassification. It is likely that some individuals with high measurements at the time of blood collection are normally exposed at low levels and *vice versa*. The higher odds ratio associating AFB1 exposure and *HPRT* mutants (19.3) versus AFB1 exposure and HCC (usually ~ 3.0) is expected. Even though most individuals in this population are exposed on some level to AFB1, only those with high levels of AFB1–albumin adducts will lead to high mutant frequencies in the *HPRT* reporter gene and even though high AFB1 exposures were not compared with truly unexposed individuals, the association suggests that only high exposures and high albumin adduct levels are associated with subsequent DNA damage severe enough to be reported by the *HPRT* gene. After all, not all individuals with AFB1 exposure as measured by AFB1 adducts will develop HCC. Therefore, not all individuals with AFB1 adducts will manifest long-term DNA damage as increased *HPRT* mutations. *HPRT* isolated from T cells allows the reporting of such damage due to its lower capacity for repair and the longer half-life. Finally, the lack of association between HBsAg and *HPRT* mutant frequency further supports the association between AFB1 exposure and *HPRT* mutant frequency. If *HPRT* mutant frequency is demonstrated to be associated with HCC,

it can possibly serve as a marker of AFB1-induced HCC, independent of hepatitis B infection-induced HCC.

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