Biomarkers in Cancer Chemoprevention
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Exposure biomarkers in chemoprevention studies of liver cancer

C.P. Wild and P.C. Turner

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, the major risk factors being hepatitis B and C viruses and aflatoxins; other factors such as alcohol are also of importance in some populations. Aflatoxin exposure biomarkers include urinary aflatoxin metabolites and aflatoxin–albumin adducts in peripheral blood. These biomarkers are well validated and have been applied in studies of many populations worldwide. They are proving to be valuable end-points in intervention studies, including chemoprevention studies. The biomarkers permit assessment of primary prevention measures to reduce aflatoxin intake. In addition, the determination of individual urinary aflatoxin metabolite profiles means that the effectiveness of chemopreventive agents designed to modulate aflatoxin metabolism can also be evaluated. Both aflatoxin–albumin adducts and urinary aflatoxin metabolites have been associated with increased HCC risk in prospective studies, indicating the predictive value of these biomarkers at the group level. However, given the multifactorial and multistep nature of HCC, it is unlikely that these exposure biomarkers will be predictive at the individual level or be of value as surrogate end-points in longer-term intervention trials aimed at reducing disease incidence. Aflatoxin-related mutations at codon 249 of the *p53* gene in plasma may be more relevant in this regard but their application requires further understanding of the temporal appearance of this biomarker in relation to the natural history of the disease.

Introduction

There are estimated to be half a million new cases of liver cancer each year worldwide. The major risk factors for hepatocellular carcinoma (HCC) are hepatitis B and C viruses (HBV and HCV), aflatoxins and alcohol. In developing countries, HBV and HCV are estimated to be associated with 67% and 24% of cases respectively, while the corresponding figures for developed countries are 29 and 22% (Pisani *et al.*, 1997). Aflatoxins contribute significantly to HCC incidence in regions of high HBV prevalence, where the two factors appear to act synergistically (Wild & Hall, 1999). Tobacco, oral contraceptives and schistosome infection also contribute to HCC incidence in some parts of the world, although they are quantitatively of less importance (Stuver, 1998). In the other major form of liver cancer, cholangiocarcinoma, the major risk factor identified is infection with liver flukes (e.g., *Opisthorchis viverrini*) (Parkin *et al.*, 1991), although *N*-nitrosamines may also play a role (Srivatanakul

et al., 1991). Occupational exposure to vinyl chloride is associated with development of another type of liver cancer, angiosarcoma (IARC, 1987). This chapter deals briefly with exposure biomarkers to the risk factors for HCC, but focuses primarily on aflatoxins, for which most experience with chemoprevention has been accrued.

Hepatitis viruses

Exposure to hepatitis B and C viruses can be accurately assessed by measuring either viral proteins or circulating antibodies to those proteins in the peripheral blood. Resolved infections and persistent active infections can also be differentiated (IARC, 1994). For example, persistent hepatitis B surface antigen (HBsAg) in the blood indicates chronic carriage and the presence of the 'e' antigen and HBV DNA indicates active viral replication. Positivity for antibody to HBsAg (anti-HBs) indicates resolved infection and immunity. Presence of antibody to hepatitis B core antigen

(anti-HBc) indicates exposure to the virus, with acute, chronic or resolved infection depending on the presence of other viral markers. In cross-sectional studies in areas of endemic HBV infection, such as Guinea-Conakry in west Africa, >80% of individuals are anti-HBs-positive and 15–20% HBsAg-positive (Sylla *et al.*, 1999). The availability of these biomarkers of hepatitis exposure has permitted the strong and specific association between HBV exposure and HCC to be established in both prospective and case–control studies (IARC, 1994). In addition, the markers can be used in evaluation of the effectiveness of HBV vaccination programmes in the prevention of both chronic carriage and HCC.

Alcohol

A number of biomarkers of exposure to alcohol consumption are being developed. One of the most promising is the detection of acetaldehyde bound to peripheral blood proteins (Conduah Birt *et al.*, 1998). Alcohol is metabolized to acetaldehyde by alcohol dehydrogenase and can be further metabolized to acetate by acetaldehyde dehydrogenase. Acetaldehyde is genotoxic and may be important in the carcinogenic action of alcohol (IARC, 1988). This is supported by recent observations that risk of alcohol-related cancer of the oral cavity and pharynx is increased in individuals with alcohol dehydrogenase genotypes associated with fast metabolism of alcohol to acetaldehyde; the risk is limited to heavy drinkers (Harty *et al.*, 1997). Consequently a biomarker of acetaldehyde–DNA or –protein adducts would be valuable. Conduah Birt *et al.* (1998) have described the covalent binding of acetaldehyde to globin and shown that the level of this biomarker is elevated in heavy drinkers. This biomarker could potentially be used to assess the impact of chemoprevention or lifestyle changes on alcohol-related cellular damage.

Aflatoxins

Measuring individual exposure to aflatoxins by either dietary questionnaire or food analysis is problematic. First, aflatoxins can contaminate a variety of cereals and oilseeds, so that measured intakes of one or two specific food commodities may not be useful as a surrogate for aflatoxin exposure, particularly in countries with a varied diet. Further, in countries with limited variety in the staple diet, the similarity of diet between individuals makes questionnaire-based assessments uninformative. Second, aflatoxin contamination is heterogeneous in nature, affecting perhaps one groundnut or maize kernel in a given batch. This hampers food analysis because it makes representative sampling extremely difficult to achieve. In the light of these difficulties, biomarkers of aflatoxin exposure have been developed and subsequently widely applied in studies of exposed human populations (Montesano *et al.*, 1997). In many ways, the aflatoxin biomarkers have become a paradigm for other chemical carcinogens and have been employed in chemoprevention studies to a degree not yet achieved in other cases (Groopman & Kensler, 1999).

The exposure biomarkers for aflatoxins have been developed on the basis of an understanding of their mechanisms of action, in particular their activation and detoxification by phase I and II enzymes (Guengerich *et al.*, 1998). The principal biomarkers of exposure include urinary metabolites and adducts, specifically albumin adducts in blood and DNA adducts in urine. These are considered briefly below; their application to chemoprevention studies is then discussed in the light of the properties of these biomarkers.

Urinary aflatoxin biomarkers

The hydroxylated aflatoxin metabolite, aflatoxin M_1 (AFM$_1$), was detected in human urine samples 30 years ago (Campbell *et al.*, 1970). Significant advances in analytical sensitivity and specificity were achieved with the application of immunoaffinity columns to purify aflatoxins from urine samples (Groopman *et al.*, 1985; Wild *et al.*, 1986b). This approach permitted a number of aflatoxins to be detected in a single urine sample (Groopman *et al.*, 1985); these included metabolites considered to be detoxification products such as AFM$_1$, but also a nucleic acid adduct of aflatoxin B_1 (AFB$_1$), AFB$_1$-N7-guanine. More recently, the AFB$_1$–mercapturic acid conjugate has been detected in human urine (Wang *et al.*, 1999).

Urinary levels of aflatoxin metabolites have been shown to correlate with measurements of dietary intake at the individual level (Zhu *et al.*, 1987; Groopman *et al.*, 1992). While this is true for a number of metabolites, it is not the case for all, aflatoxin P_1 being one exception. This is probably

because this metabolite can be excreted in both the bile and urine and hence measurements in only one compartment can be misleading (Groopman *et al.*, 1993). However, excellent correlations between dietary intake, AFB_1-N7-guanine and total urinary aflatoxin levels have been found, particularly when exposure and urinary levels are integrated over a number of days by collecting consecutive 24-hour urine samples (Groopman *et al.*, 1992). This latter approach overcomes problems due to the rapid excretion of aflatoxins in the urine, which occurs over some 24–48 hours following ingestion and can lead to rapidly fluctuating levels. For example, in a study in The Gambia, 24-hour urine samples were collected on four consecutive days from 20 individuals and the day-to-day fluctuation in urinary levels was marked, exceeding two orders of magnitude in some cases (Groopman *et al.*, 1992; Hall & Wild, 1994). This could clearly lead to misclassification of exposure status if only a single day's level were taken into account. However, when the mean daily urinary aflatoxin levels over four days were compared with the mean daily aflatoxin intake for each individual over one week, excellent correlations were observed for both total urinary aflatoxins and AFB_1-N7-guanine (Groopman *et al.*, 1992).

In a prospective cohort study in Shanghai, urinary levels of aflatoxin metabolites were positively associated with HCC risk and a synergistic interaction between HBV and aflatoxins was observed (Qian *et al.*, 1994). The association was strongest with AFB_1-N7-guanine adduct, but was also positive with AFM_1. Similar data have been obtained from a prospective cohort study in Taiwan using urinary aflatoxins and aflatoxin–albumin adducts as exposure markers (Wang *et al.*, 1996). To date, the biomarker data in these studies have been used as a categorical rather than continuous variable, with simple dichotomization into positive or negative or high and low levels. Thus no quantitative dose–response data are yet available.

Aflatoxin–albumin adducts
Aflatoxin–albumin adducts are detected in peripheral blood of both animals and humans exposed to aflatoxin. As with DNA adducts, there is a linear dose–response relationship following aflatoxin exposure in rats (Wild *et al.*, 1986a, 1996).

Furthermore, with multiple exposures, adducts accumulate and reach a plateau; in different strains of rats, mice, hamsters and guinea-pigs, the albumin adducts are correlated with hepatic DNA adduct levels and reflect qualitatively the susceptibility of these four species to aflatoxin toxicity and carcinogenicity (Wild *et al.*, 1986a; 1996). In rats, the aflatoxin–albumin conjugate appears to turn over with the half-life of albumin itself (Sabbioni *et al.*, 1987; Wild *et al.*, 1986a). This has not been formally demonstrated in humans, but assuming it to be the case, a single measurement should reflect an integration of exposure over the previous two to three months. Certainly, the albumin adduct measurement provides a more stable biomarker than urinary metabolites (Hall & Wild, 1994). Aflatoxin–albumin adducts were associated with increased HCC risk in HBV carriers in the Taiwan cohort mentioned above (Wang *et al.*, 1996).

Aflatoxin–albumin adducts have proved to be an excellent marker for assessing exposure in many countries worldwide (Montesano *et al.*, 1997). We detected the highest levels and prevalence of exposure in west Africa (The Gambia, Guinea, Senegal, Burkina Faso) where groundnuts, a frequent source of aflatoxin exposure, are consumed as a dietary staple. High levels are also observed in maize-consuming countries such as Kenya and parts of southern China. Lower levels of exposure occur where the diet is more varied (e.g., Thailand, Nepal). In contrast, in Europe, the United States and Canada, almost no human sera have been found to be aflatoxin-positive in our studies (Montesano *et al.*, 1997; Wild *et al.*, unpublished data).

If adducts are to be used as outcome markers in intervention studies, it is important to understand the parameters which can influence their level. In a study of environmental and genetic determinants of aflatoxin–albumin adduct levels in The Gambia, we observed that season and geography were major factors associated with the levels of adducts measured (Wild *et al.*, 2000). The seasonal variations were consistent with previous observations (Wild *et al.*, 1990) but were probably further complicated by annual variations in toxin levels dependent on climatic conditions. In contrast, genetic polymorphisms in aflatoxin-metabolizing enzymes or phenotypic expression of cytochrome P450 3A4 (measured by urinary cortisol:6-hydroxy-

cortisol ratio) were not strong determinants of adduct levels (Wild *et al.*, 2000). In adults, HBV status did not appear to be associated with higher adduct levels, although there was a suggestion of this in children, particularly at the time of acute infection (Allen *et al.*, 1992; Wild *et al.*, 1993; P.C. Turner, M. Mendy, A.J. Hall, M. Fortuin, H. Whittle & C.P. Wild, unpublished data).

p53 mutations

The AFB1-N7-guanine adduct can give rise to G to T transversion mutations (Bailey *et al.*, 1996). In HCC from regions of high aflatoxin exposure, *p53* mutations are common and specifically a high prevalence of an AGG (Arg) to AGT (Ser) mutation at codon 249 has been detected (Montesano *et al.*, 1997). This mutation is also seen in non-tumour liver tissue from regions of the world where aflatoxin exposure is elevated (Aguilar *et al.*, 1994), but is extremely rare in HCC from countries where aflatoxin exposure is low, even though HBV infection is present in association with many of the tumours. Harris has concluded that the weight of evidence, according to the Bradford Hill criteria, supports the causality of the relationship between aflatoxin exposure and the codon 249 mutation (Hussain & Harris, 1998). Kirk *et al.* (2000) have reported the presence of this same mutation in the plasma of HCC cases in The Gambia. The prevalence in cases (36%) was significantly higher than in cirrhotics (15%) and controls without liver disease (6%). Furthermore, the mutation was not detected in plasma from HCC cases from France. It remains to be determined whether the codon 249 mutations in plasma DNA observed in The Gambia are related specifically to the presence of HCC, in which case the cirrhotics and controls who were positive for the biomarker may have occult cancer, or whether the mutation also reflects heavy aflatoxin exposure with frequent somatic mutation and clonal expansion of individual hepatocytes. A prospective study would provide an answer to this question.

Chemoprevention

HBV vaccination is a priority for reducing the global burden of HCC. Currently, however, only about 1% of African children receive the vaccine. There are 360 million HBV carriers worldwide 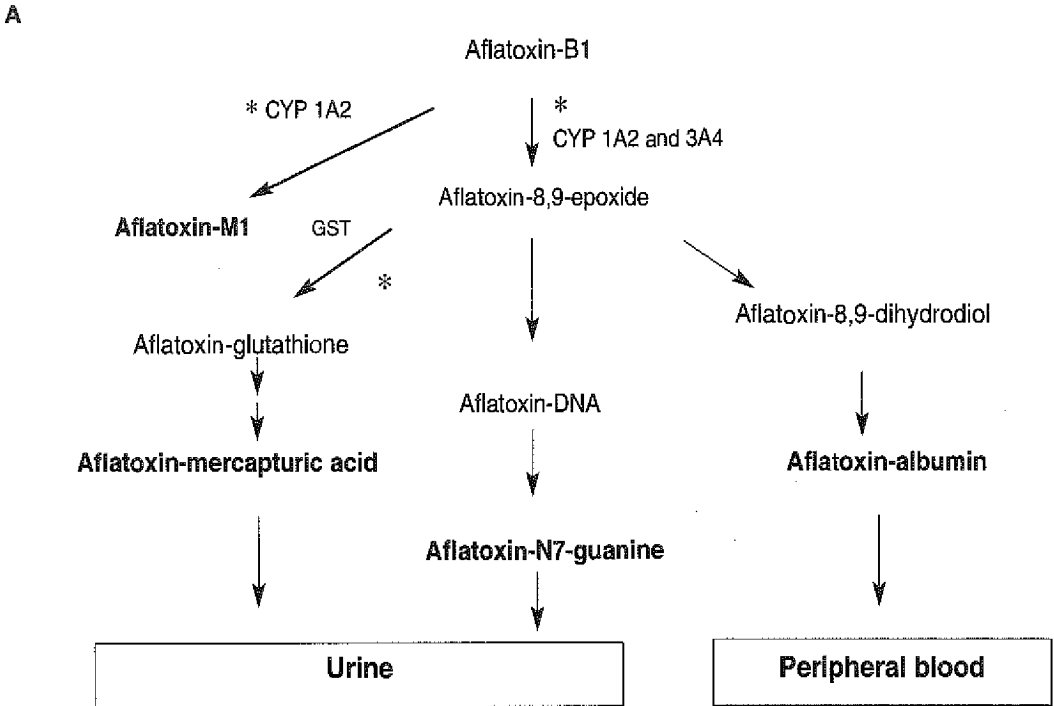and the continuing restricted access to the vaccine means that the number of carriers will remain high for at least several decades. Given the high numbers of HBV carriers and the synergistic interaction between aflatoxins and HBV, intervention to reduce aflatoxin exposure is also merited.

Interventions to reduce aflatoxin-related disease involve initiatives at the individual level or community level (Wild & Hall, 2000). The community level approach can involve either pre- or post-harvest measures, while at the individual level the intervention can comprise a change in diet to avoid intake of frequently contaminated foods or chemoprevention to reduce the toxicity of aflatoxins once ingested.

Aflatoxin exposure biomarkers have been developed based on an understanding of the metabolism of these compounds in humans (Figure1 A). A number of these biomarkers have been validated and subsequently widely applied in human populations. Modulation of the level of a biomarker can be used to assess the effectiveness of interventions (Figure 1 B). For example, primary prevention measures aimed at reducing aflatoxin intake should lead to a reduction in all biomarker levels. In addition, the ability to examine individual aflatoxin metabolite profiles means that the effectiveness of chemopreventive agents designed to modulate aflatoxin metabolism can also be evaluated (Figure 1 B).

Most progress has been made using the urinary aflatoxin metabolites and aflatoxin–albumin adducts in relation to chemoprevention with oltipraz in the People's Republic of China, but the latter biomarker is also being applied in a post-harvest intervention in Guinea-Conakry (Sylla *et al.*, 1999). The use of biomarkers in these studies is discussed briefly below.

Animal species show marked differences in sensitivity to aflatoxin–DNA and –protein adduct formation and susceptibility to aflatoxin carcinogenesis (Wild *et al.*, 1996). Induction of glutathione S-transferases (GST) and aflatoxin aldehyde reductase decreases aflatoxin–DNA and –protein adduct formation and blocks aflatoxin carcinogenicity in rats (Judah *et al.*, 1993; Groopman & Kensler, 1999). Therefore a similar modulation of the balance between aflatoxin activation and detoxification in humans has been sought. The drug used, oltipraz, is one originally prescribed to treat schistosomiasis.

A

Aflatoxin-B1

＊ CYP 1A2

＊
CYP 1A2 and 3A4

Aflatoxin-8,9-epoxide

Aflatoxin-M1   GST

＊

Aflatoxin-8,9-dihydrodiol

Aflatoxin-glutathione

Aflatoxin-DNA

Aflatoxin-mercapturic acid

Aflatoxin-albumin

Aflatoxin-N7-guanine

| Urine | Peripheral blood |

B

Aflatoxin biomarker	Primary intervention:	Secondary intervention:	
	Reduce intake	Oltipraz	Chlorophyllin
Aflatoxin–albumin	Decrease	Decrease	Decrease
Aflatoxin M_1	Decrease	Decrease	Decrease
Aflatoxin-N7-guanine	Decrease	Decrease	Decrease
Aflatoxin–mercapturic acid	Decrease	Increase	Decrease

Figure 1. Aflatoxin metabolism and biomarkers

A: Aflatoxin biomarkers in bold, oltipraz effects indicated by stars. Oltipraz reduces CYP 1A2 and increases glutathione S-transferase (GST) activity (Wang et al., 1999) (see text).

B: Effects of various aflatoxin intervention strategies on biomarker levels. Primary intervention to reduce aflatoxin intake, secondary intervention by (a) oltipraz to modify aflatoxin metabolism, (b) chlorophyllin to reduce gastrointestinal absorption (see text for details).

In China, Kensler and colleagues have demonstrated that oltipraz can modulate aflatoxin metabolism, probably by inhibiting activity of cytochrome P450 (CYP) 1A2, an enzyme which activates AFB_1 to AFB_1-8,9-epoxide and by increasing the level of GST-mediated conjugation of the epoxide to glutathione (Jacobson et al., 1997; Kensler et al., 1998, 1999; Wang et al., 1999; see also Kensler et al., this volume). These effects were demonstrated by assay of urinary AFM_1 (a product of CYP1A2 metabolism of AFB_1), peripheral blood aflatoxin–albumin adducts and the urinary

aflatoxin-mercapturic acid conjugate. In a phase IIb clinical trial in Qidong County, People's Republic of China, 250 or 500 mg oltipraz was administered weekly over one year (Kensler *et al.*, 1999). While the phase II trials establish an effect of oltipraz on aflatoxin metabolism, a further trial would be required to evaluate the chemopreventive action of oltipraz against aflatoxin-induced carcinogenesis, with disease incidence as an outcome. Unless aflatoxin exerts a hepatocarcinogenic effect late in the natural history of the disease, a long follow-up would be required to detect the effect of the intervention. Oltipraz has been reported to inhibit HBV replication in cells *in vitro* by induction of a p53-mediated effect on the HBV reverse transcriptase (Chi *et al.*, 1998). If this also occurs *in vivo*, markers of HBV replication could be used to monitor the effect in oltipraz intervention trials.

If any of the above aflatoxin biomarkers are demonstrated to be strong predictors of cancer risk, they could be used as surrogate measures of disease outcome in future studies. It is unlikely that the transient aflatoxin adducts (with DNA or albumin) will fulfil this requirement at the individual level; this is suggested indirectly in rats where a correlation between adducts and liver cancer was seen at the group but not individual level (Kensler *et al.*, 1997). However, the specific *p53* codon 249 mutation related to aflatoxin exposure may be more predictive of individual risk. In this respect, the above-mentioned recent identification of this mutation in the plasma of Gambians with liver cancer or cirrhosis is encouraging (Kirk *et al.*, 2000).

In addition to oltipraz, a number of other chemopreventive agents are being developed with respect to aflatoxin (Kelly *et al.*, 2000). One of these is chlorophyllin, which can inhibit aflatoxin adduct formation and carcinogenicity by non-covalent complex formation between the two compounds (Hayashi *et al.*, 1999). The biomarkers of aflatoxin exposure mentioned above would be equally applicable to evaluating effects of this type of chemopreventive agent.

In summary, both aflatoxin–albumin adducts and urinary aflatoxin metabolites have been associated with increased HCC risk in prospective studies, indicating the predictive value of these biomarkers at the group level. However, given the multifactorial and multistep nature of HCC, it is unlikely that these exposure biomarkers will be predictive for HCC risk at the individual level or be of value as surrogate end-points in longer-term intervention trials aimed at reducing disease incidence. The aflatoxin-related codon 249 *p53* mutations in plasma may be more relevant in this regard, but further understanding of the temporal relationship between the appearance of this biomarker and the natural history of the disease is needed.

Acknowledgement

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Corresponding author:

C.P. Wild
Molecular Epidemiology Unit,
Epidemiology and Health Services Research,
School of Medicine,
Algernon Firth Building,
University of Leeds,
Leeds LS2 9JT,
UK