



Mini-review

A model of interaction: Aflatoxins and hepatitis viruses in liver cancer aetiology and prevention

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and has an extremely poor prognosis. The majority of cases occur in south-east Asia and sub-Saharan Africa where the major risk factors are chronic infection with hepatitis B and C viruses (HBV and HCV) as well as dietary exposure to aflatoxins. Aflatoxin B₁, the most commonly occurring and potent of the aflatoxins is associated with a specific AGG to AGT transversion mutation at codon 249 of the p53 gene in human HCC, providing mechanistic support to a causal link between exposure and disease. Prospective epidemiological studies have shown a more than multiplicative interaction between HBV and aflatoxins in terms of HCC risk. However, the biology underlying this statistical interaction is not fully understood. There are a number of potential mechanisms including, among others: the fixation of AFB₁-induced mutations in the presence of liver regeneration and hyperplasia induced by chronic HBV infection; the predisposition of HBV-infected hepatocytes to aflatoxin-induced DNA damage; an increase in susceptibility to chronic HBV infection in aflatoxin-exposed individuals; and oxidative stress exacerbated by co-exposure to aflatoxins and chronic hepatitis infection. Priorities for prevention are global HBV vaccination, primary and secondary prevention strategies against aflatoxin and the avoidance of transmission of HCV through good hygiene practices.

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1. Introduction

More than 600,000 people die each year from liver cancer. The majority of cases are hepatocellular carcinomas (HCC) and most occur in developing countries (Table 1), particularly China, other parts of south-east Asia and sub-Saharan Africa [1]. This cancer is more common in men than women, although sex ratios vary by region [2]. The number of new liver cancer cases each year is roughly equivalent to the number of deaths, illustrating the poor access to, and efficacy of, treatment.

In the current review we briefly discuss the role of the most common causes of HCC in developing countries, namely hepatitis viruses and aflatoxins in relation to the

natural history of the disease and the implementation of preventive strategies. Other risk factors of relevance, mainly in developed countries, including alcohol [3] and tobacco [4,5] have been reviewed elsewhere [2].

2. Epidemiology of hepatitis viruses and HCC

Following the seminal study by Beasley et al. [6] many sero-epidemiological studies have demonstrated that chronic active infection with hepatitis B virus (HBV) is a risk factor for this cancer [7]. Some 360 million people, 5% of the world population, is chronically infected with HBV. The fact that HBV infection and associated liver disease can be prevented by vaccination is a significant achievement in the field of cancer prevention. Goldstein et al. [8] calculates that without HBV vaccination, among the global birth cohort born in the year 2000 there would

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Table 1

Estimated cases of liver cancer and number attributable to HBV or HCV.

	Liver cancer cases	HBV attributable cases	HCV attributable cases
Developed countries	110,800	26,000	22,000
Developing countries	515,300	303,000	172,000
World	626,100	340,000	195,000

Taken from [1].

be almost 1.5 million HBV-related deaths, mainly due to cirrhosis and HCC.

An estimated 170 million people worldwide are chronically infected with hepatitis C virus (HCV) and epidemiological studies have demonstrated an association with increased HCC risk, leading to its classification as a human carcinogen by the International Agency for Research on Cancer (IARC) [2,7]. The pattern of HCC mortality associated with the HCV epidemic revealed that the natural history of HCV infection and its association with HCC mortality varies considerably in different regions of the world [9,10]. A recent comprehensive review of 27,881 HCC cases from 36 countries in relation to the presence of hepatitis infection showed widely varying prevalence of HBV and HCV [11]. Some countries such as Egypt, Japan, Pakistan and Mongolia had a particularly high number of cases associated with HCV (40–69%). HCV and HBV coinfection in HCC cases tended to be relatively infrequent in all regions (<10%), although Mongolia was a notable exception.

The fraction of HCC cases attributable to HBV and HCV has been estimated to be 23% and 20% in developed countries and 59% and 33% in developing countries [1].

3. Epidemiology of aflatoxins and HCC

Aflatoxins, naturally occurring secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*, are common contaminants of a variety of staple foods such as maize and groundnuts in developing countries [12,13]. A significant portion of aflatoxin contamination occurs during storage under conditions that promote fungal growth and toxin production. IARC has classified naturally occurring aflatoxins as human carcinogens [12,14,15]. Aflatoxin B1 (AFB1) is the most common and potent of the aflatoxins. It is metabolised predominantly in the liver to an AFB1-8,9-*exo*-epoxide which forms a promutagenic AFB1-N7-guanine DNA adduct that results in G to T transversion mutations [13]. In human HCC from areas of high aflatoxin exposure up to 50% of tumours have been shown to harbour a specific AGG to AGT point mutation in codon 249 of the TP53 tumour suppressor gene (codon 249^{ser} mutation) [13,16].

Ecological studies in the 1970s and 1980s in sub-Saharan Africa and south-east Asia reported correlations between aflatoxin levels in crops or food and HCC rates [15]. Unfortunately the majority of this early work did not take account of HBV infection and aflatoxin exposure was not measured at the individual level [14,15]. In the 1990s significant advances were made in understanding the role of aflatoxins as a risk factor for HCC, particularly

in relation to joint effects with chronic HBV infection. Progress came from two principle sources: prospective cohort studies and analyses of the molecular pathology of HCC specimens, both of which involved aflatoxin biomarkers.

Two key cohort studies in Asia and a large case-control study in Africa employed biomarkers (urinary aflatoxin metabolites, blood aflatoxin–albumin adducts or codon 249^{ser} mutations) to improve individual aflatoxin assessment and showed significant interactions with chronic HBV infection in relation to HCC risk [17–19]. Both studies in Asia, Shanghai [17–19] and Taiwan [18], reported a more than multiplicative interaction between the two risk factors. In a follow-up of the Taiwan cohort, Sun and co-workers [20] showed that in HBsAg carriers, those with detectable aflatoxin–albumin adduct were more likely to develop HCC. In similar fashion other studies restricted to individuals chronically infected with HBV revealed increased HCC risk in those also positive for aflatoxin biomarkers [21–23]. In a case-control study in The Gambia, the codon 249^{ser} mutation was examined in the plasma of HCC cases, cirrhosis patients and controls. The presence of both the codon 249^{ser} mutation and HBV infection was associated with an OR = 399 (95% CI: 48.6–3270) [24,25]. A comprehensive review of studies of HBV and aflatoxins in The Gambia has been published [26]. These studies and others of HBV and aflatoxin, suggesting an interaction between the two, are summarised in Table 2. Other case-control studies in Africa have been reported, for example in Sudan [27] where peanut consumption (as a surrogate for aflatoxin exposure) was associated with HCC and showed a more than additive interaction with HBV. There are no prospective cohort studies of aflatoxins, HBV and HCC in Africa to date.

In comparison to the study of aflatoxins and interaction with HBV there has been little focus on the potential for interaction with HCV infection [28,29]. One recent study in Taiwan suggested that aflatoxin–albumin adducts were associated with more advanced liver disease in individuals infected with HCV [29]. It will be of interest in the future to study potential interaction in populations such as this where HBV infection may be controlled by vaccination but where aflatoxins and HCV infection persist. It should be noted, however, that whilst HBV infection occurs most commonly in developing countries early in life, infection with HCV is normally much later, thus affecting the time period over which aflatoxins and HCV may interact.

4. Animal carcinogenicity

Several animal species have been examined for AFB1-induced mutations at the equivalent to codon 249 in hu-

Table 2
Studies of the interaction between aflatoxins and HBV in HCC.

Population [Reference]	Cohort	Cases	Controls	Biomarker	OR
Shanghai, PRC [17]	18,224 Males	50	267	Urinary AF biomarker ^d	3.4 (1.1–10.0) AF alone 7.3 (2.2–24) HBsAg alone 59.4 (16.6–212) AF and HBsAg
Taiwan [18]	12,040 Males 13,758 Females	56	220	Urinary AF metabolites ^e	1.7 (0.3–10.8) AF alone 22.8 (3.6–143.4) HBsAg alone 111.9 (13.8–905) AF and HBsAg
Taiwan [18]	As above	29 HBsAg +ve	21 HBsAg +ve	Urinary AF metabolites ^f	5.5 (1.3–23.4)
Taiwan [23]	4691 Males 1796 Females	33 (20) ^g	123 (86) ^g	AF-albumin	5.5 (1.2–24.5) AF alone 129 (25–659) AF and HBsAg
Taiwan [21]	4841 Male HBsAg carriers 2501 Male non-carriers	43 HBsAg +ve	86 HBsAg +ve	Urinary AFM1	6.0 (1.2–29.0) ^h
Taiwan [20]	12,024 Males 13,594 Females	79 HBsAg +ve	149 HBsAg +ve	AF-albumin	2.0 (1.1–3.7) ^b
Qidong Co., PRC [22]	145 Male HBsAg carriers	22 HBsAg +ve	123 HBsAg +ve	Urinary AFM1 ^c	3.3 (1.2–8.7)

^a Highest compared to lowest tertile of AFM1 level; adjusted for educational level, ethnicity, alcohol, cigarette smoking.

^b Detectable versus non-detectable; adjusted for sex, age and residence.

^c Eight monthly urine samples were collected over follow-up and urinary AFM1 analysis was conducted on a pooled sample; AFM1 positive compared to negative.

^d Presence versus absence of any aflatoxin biomarker; adjusted for cigarette smoking.

^e Low versus high urinary aflatoxin biomarker; adjusted for cigarette smoking and alcohol drinking.

^f Low versus high urinary aflatoxin biomarker; adjusted for age, residence, cigarette smoking and alcohol drinking.

^g Only the numbers of subjects in brackets had samples for analysis of aflatoxin biomarker.

Table 3
p53 Mutations in animal tumours at codon 249 or equivalent (updated from [30]).

Species	Nucleotide sequence comparison ^a			Prevalence of codon 249 mutation	Reference
	248	249	250		
Human	CGG	AGG ^b	CCC		
Monkey	CGC	AGG	CCC	0/4	[31]
Rat					
Hyperplastic nodules	CGC	CGG	CCC	0/6	[81]
Focal lesions and tumours				1/17	[82]
Woodchuck	CGA	CGG	CCC	0/8	[83]
Ground squirrel	CGC	CGG	CCC	0/7	[84]
Duck	CGT	CGC	CCA	0/15	[35]
Tree shrew	CGG	CGC	CCC	0/5	[85]
Mouse	CGC	CGA	CCT	0/19 ^d	[43]
Mouse – HUPKI ^c	CGG	AGG	CCC	0/36 ^d	[43]

^a The p53 gene codon numbering refers to the human p53 gene equivalent.

^b Guanine targeted by aflatoxin in the human p53 sequence.

^c HUPKI – human p53 knock-in mouse strain [86].

^d 19 and 36 liver masses from 14 and 19 mice, respectively, were analysed.

mans (Table 3; [30]). No G to T transversions at this target were found in tumours from rats, tree shrews or ducks. However, two major limitations to this comparison are the species differences in DNA sequences (see Table 3) and the small number of tumours analysed. In monkeys the codon 249 sequence is the same as humans but data for only four HCC (two from one animal) were reported [31]. It is noteworthy that the studies in ducks, woodchucks and tree shrews did include some animals also infected with hepadnaviruses in addition to aflatoxin treatment.

Different animal models have been used to examine the interaction between hepatitis virus and AFB1 exposure, but in general these have suffered from a number of limitations (see [32]). For example, in ducks infected with duck hepatitis B virus there is little liver pathology associated with infection, possibly reflecting the absence of the HBx

gene [33]. In fact liver tumours in DHBV infected ducks were only reported when co-exposure to AFB1 occurred [34,35]. In woodchucks there is more liver damage associated with woodchuck hepatitis infection and liver cancer is induced in the absence of co-exposure to AFB1 [36]. Studies of the interaction with AFB1 involved relatively few animals, although some evidence for increased liver tumour occurrence was reported with both exposures [37]. There was also a report of a higher risk of liver tumours in tree shrews (*Tupaia belangeri chinensis*) exposed to hepatitis infection and AFB1, compared to either agent alone [38].

An alternative to these models of hepadnavirus infection in a natural host is a variety of HBV transgenic and knockout mouse lineages. These include mice expressing various HBV antigens (e.g., HBsAg, HBeAg and HBx) with some being additionally engineered to be p53 deficient or

to have a p53ser246 mutant (this mutation in mice mimics the human codon 249^{ser} mutation) [39,40]. HBV transgenic mice overexpressing HBsAg in the liver showed more HCC than non-transgenic littermates when exposed to AFB1 [41]. In later work in a series of different transgenic lineages given AFB1, the highest incidence of liver tumours was observed in mice with the p53ser246 mutation and functional p53 (Table 4). In addition these data indicate that HBsAg-associated cell injury, wild-type p53 expression, male gender and AFB1 exposure are relevant to HCC development. In transgenic mice expressing the HBx gene, AFB1 treatment induced significantly more liver tumours than in wild-type mice [42].

Experiments by Tong et al. [43] in Hupki mice, in which exons 4–9 of the mouse p53 were replaced by the corresponding human p53 exons in the germ-line, revealed an increase in HCC after AFB1 treatment, but the codon 249^{ser} mutation was not detected. However, in this latter strain, unlike the studies of Sell and colleagues, there was no expression of HBV antigens [40]. It is conceivable that the presence of functional active HBsAg is required in the selection of a specific liver cell population (putative liver stem cells) containing the mutated p53 gene.

5. Potential mechanisms of interaction

The epidemiological studies mentioned above clearly suggest a statistical interaction between HBV and AFB1 in the induction of HCC. However, a better understanding of the biological mechanisms underlying this interaction is of importance in designing more effective prevention measures and in screening for early HCC.

Sero-epidemiological studies have clearly shown that in the natural history of HBV infection two factors of importance in determining the risk of HCC are the age at primary infection and the presence of HBeAg or HBV DNA, biomarkers of active viral replication, in patients with chronic active hepatitis [44]. In addition, HBx protein affects various cellular functions relevant to cancer development, namely p53 and oxidative DNA damage [16]. These observations are important when exploring potential mechanisms of interaction with aflatoxins.

As is well documented, the development of HCC results from the accumulation of various genetic changes at different stages of liver carcinogenesis [45]. In recent years considerable evidence has accumulated supporting the view that HBV has a direct role in the induction of HCC. Namely it has been shown [46,47] that HBV integration is a fre-

quent event occurring at all stages of infection, with some common sites of integration in cellular genes, and it precedes tumour development.

Chronic liver injury and regenerative hyperplasia, resulting from HBV infection, are critical to the development of liver cancer [16,48]. It is possible that aflatoxin-induced DNA adducts are fixed as mutations due to the HBV-related increase in cell proliferation and hyperplasia, thus promoting the clonal expansion of mutant cells. Inflammation and oxidative stress associated with chronic active hepatitis may also result in DNA damage and mutations. To date observations in human populations are somewhat contradictory in terms of the association between HBV infection and biomarkers of oxidative stress [49–51]. Nevertheless, in a study directly assessing 8-oxo-deoxyguanosine in human liver both HBV and HCV infection were associated with DNA damage [52]. There was a greater effect seen in association with the latter virus, possibly reflecting the excessive iron deposition seen in chronic HCV infection. In two recent studies correlations were reported between biomarkers of aflatoxin exposure and oxidative stress [49,50]. This correlation may be indicative of AFB1 exposure itself inducing oxidative stress, but it is also possible that oxidative stress from other causes alters AFB1 metabolism and hence biomarker levels.

HBV could predispose hepatocytes to the carcinogenic action of aflatoxins. For example, human liver epithelial cells, expressing wild-type p53 and transfected with HBx gene were more sensitive to the cytotoxic action of AFB1-8,9-epoxide than were the parent cells [53]. The HBx expressing cells were also more prone to apoptosis and to induction of mutations at codon 249 of the p53 gene. One possible explanation is that HBx could inhibit excision repair thus leading to increased AFB1 DNA adduct persistence and mutation induction [16,54]. HBV may also alter the hepatic expression of aflatoxin metabolising enzymes and consequently the extent to which aflatoxins bind to DNA. In woodchucks the results have been somewhat contradictory [55,56]. However, studies in HBV transgenic mice revealed an induction of specific cytochrome P450s (CYP) in association with liver injury induced by overexpression of HBsAg [57,58]. The absence of CYP induction in transgenic mice expressing only HBx protein argues against transactivation of the CYP genes by this protein [59].

The effects of liver injury are not limited to CYP enzymes. An increase in GST pi was also observed in the HBV transgenic mice [60] and in HepG2 cells that were HBV-transfected, whilst expression of GST α class enzymes was significantly decreased. Transfection of the HBx gene into these latter cells also decreased the amount of GST α class protein [61]. In human liver, GST activity was lower in the presence of HBV DNA [62] suggesting viral infection may compromise the ability of hepatocytes to detoxify chemical carcinogens. Overall, the effects of HBV infection on aflatoxin metabolism are likely to be complex, but there is potential for an altered balance of activation and detoxification of carcinogens during the natural history of an infection.

Aflatoxin exposure may alter the effects of the hepatitis virus, perhaps affecting susceptibility to infection or viral replication. In ducklings for example, AFB1 treatment re-

Table 4

Carcinogenic response after AFB1 treatment in male transgenic mice expressing HBsAg, p53ser246 and p53 proteins.

HBsAg	p53ser246	p53	Mice with nodules >1 cm (%)
+	+	+/+	100
+	–	+/+	68.8
–	+	+/+	14.2
–	+	+/-	71.4
–	–	+/-	28.5
–	–	+/+	10.0

Adapted from [40].

sulted in a significant increase in duck HBV DNA (in serum and liver) and viral RNA and duck HBV large envelope protein in liver [63]. Consistent with this, HepG2 cells transfected with re-circularised HBV and treated with AFB1 showed a 2–3-fold increase in HBsAg 96 h post-treatment [64]. DNA damage can also increase viral DNA integration into the host genome [65] and it is possible that AFB1 could exert this effect directly, or indirectly via the oxidative stress mentioned above. Finally AFB1 is known to be immunosuppressive in animals and may affect susceptibility to chronic viral infection in exposed individuals [66].

6. Implications for prevention

HBV vaccination is a priority for reducing the global burden of HCC. Currently, only about half of children in the WHO African region are estimated to receive the vaccine [67]. Kirk et al. [26] discuss the efficacy of HBV vaccination in various regions of the world in reference to the differing natural history of HBV infection. For HCV, in the absence of a vaccine, good hygiene practices e.g., use of clean needles, are vital [68]. Given the estimated 360 million HBV carriers worldwide and the synergistic interaction between aflatoxins and HBV, interventions to reduce aflatoxin exposure are also merited (see [69,70]). This is of even greater relevance when the potential adverse health effects of aflatoxin exposure on child growth and immunity are considered [66,71,72].

In some circumstances a shift in diet, away from commodities commonly contaminated by aflatoxins, can lead to a reduction in exposure. An example of this is in China where economic developments have resulted in reduced maize consumption [73]. In terms of limiting aflatoxin levels in foods, pre-harvest would be the most effective point of control including various measures to reduce crop stress (e.g., improved irrigation, use of fungicides, pesticides and insecticides, use of strains resistant to fungal colonisation, biocontrol by introduction of competitive non-aflatoxigenic strains of *A. flavus* and genetically modified crops that inhibit fungal colonisation) [69]. However, these approaches can be expensive and are of limited applicability at present at the subsistence or small-farm level.

Aflatoxins often accumulate during food storage making the control of post-harvest storage conditions a vital component in limiting aflatoxin levels. In a primary prevention study in Guinea, aimed at groundnut storage, an approximately 60% reduction in aflatoxin–albumin adducts was seen in subjects consuming groundnuts in the intervention villages compared to controls at 5 months post-harvest [74]. This study suggests that simple, inexpensive approaches can offer significant benefits at the small-farm level.

An alternative to the above primary prevention has been to try and modify the effects of aflatoxins once ingested, either by preventing absorption or by modifying metabolism. The use of clays to absorb aflatoxins and prevent bioavailability has been demonstrated in animals and has recently been extended to trials in exposed people [75]. This study showed a reduction in both aflatoxin–albumin adducts and urinary AFM1 in Ghanaian subjects taking the clay-filled capsules over a 3-month period.

With respect to altered metabolism, a number of approaches have been explored [70]. Chlorophyllin may act both to reduce absorption but also to modify aflatoxin metabolism. This compound has been tested in a chemoprevention trial in China and resulted in a 55% reduction in urinary AFB1–N7–Gua compared to those taking placebo [76]. Oltipraz is effective in blocking both aflatoxin DNA adduct formation and hepatocarcinogenesis in animal models by modifying the level of detoxifying enzymes such as GSTs. An increase in the urinary excretion of the aflatoxin–mercapturic acid conjugate (AFB–NAC) and a decrease in AFM1 have been demonstrated in a clinical trial in aflatoxin-exposed individuals [70]. Green tea polyphenols were applied in a 3-month intervention trial, also in China, and a reduction in both urinary AFM1 and aflatoxin–albumin adducts and an increase in urinary AFB–NAC was observed, consistent with an inhibition of bioactivation and an increase in detoxification [77]. Finally a chemoprevention trial using a broccoli sprout extract did not show reduction in urinary AFB1–N7–Gua excretion, probably due to the unexpected inter-individual variation in bioavailability of dithiocarbamates from the broccoli. However, when a comparison was made at the individual level between bioavailable dithiocarbamate and AFB1–N7–Gua there was a strong inverse association [78].

7. Conclusions

Overall the majority of HCC cases in developing countries are associated with hepatitis viruses and aflatoxins. The vaccine against HBV offers a major opportunity to reduce HCC incidence in the coming years. Given that the effects of aflatoxins are most evident among chronic HBV carriers, the vaccination will also reduce the adverse consequences of these commonly occurring toxins. Nevertheless, the facts that: (1) comprehensive vaccine introduction in HBV endemic areas will take time; (2) current chronic HBV infection rates in adults remain at 10–20% in many populations, and (3) there are potential additional detrimental effects of aflatoxins on child growth as well as occasional severe acute poisoning outbreaks [79], imply that efforts to reduce aflatoxin exposure should also be pursued. In addition, the economic costs of aflatoxin spoilage of crops will indirectly have an adverse effect on human health. This latter point highlights the need to consider the economic and health costs associated with aflatoxins in the broader context of agriculture and health [80].

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