2 Environmental Carcinogens and Risk for Human Liver Cancer

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ABSTRACT

Collectively liver cancer, including hepatocellular carcinoma (HCC) and cholangiocarcinoma, accounts for 5.7% of all reported cancer cases and is the sixth most common cancer diagnosed worldwide. The incidence of liver cancer varies enormously globally and unfortunately the burden of

B.I. Carr (ed.), *Hepatocellular Carcinoma*, Current Clinical Oncology
DOI 10.1007/978-1-60327-376-3_2

this nearly always fatal disease is much higher in the economically less developed countries of Asia and sub-Saharan Africa. This chapter will review the significant data that link exposures to specific environmental carcinogens and the development of HCC in many parts of the world. These epidemiologic studies have been made possible by devising biomarkers reflective of exposure and risk. The translation of these basic science findings to an understanding of the etiology of HCC has also provided guidance for the development of preventive interventions in high-risk populations. Thus, the consistency of the experimental animal and human data points to the important role that environmental exposures play in gender differences in HCC risk.

Key Words: Hepatocellular carcinoma (HCC); Cholangiocarcinoma; aflatoxin B_1 (AFB_1); environmental exposures; biomarkers; hepatitis B surface antigen (HBsAg); hepatitis B virus (HBV); hepatitis C virus (HCV)

1. INTRODUCTION

Collectively liver cancer, including hepatocellular carcinoma (HCC) and cholangiocarcinoma, accounts for 5.7% of all reported cancer cases and is the sixth most common cancer diagnosed worldwide *(1)*. The incidence of liver cancer varies enormously globally and unfortunately the burden of this nearly always fatal disease is much higher in the economically less developed countries of Asia and sub-Saharan Africa (Fig. 1) *(2)*. HCC is also the most rapidly rising solid tumor in the United States and is overrepresented in minority communities, including African-Americans, Hispanic/Latino-Americans, and Asian-Americans *(3)*. Overall, there are more than 650,000 new cases each year and over 200,000 deaths annually in the People's Republic of China (PRC) alone *(4, 5)*. In contrast with most common cancers in the economically developed world where over 90% of cases are diagnosed after the age of 45, in high-risk regions for liver cancer onset begins to occur in both men and women by 20 years of age and peaks between 40 and 49 years of age in men and between 50 and 59 years of age in women *(1, 6, 7)*. This earlier onset of HCC might be attributable to exposures that are both substantial and persistent across the life span. Gender differences in liver cancer incidence have also been described and the worldwide annual age-standardized incidence rate among men is 15.8 per 100,000 and 5.8 per 100,000 among women *(8)*. These epidemiologic findings are also similar to experimental animal data for one potent liver carcinogen linked to human HCC, aflatoxin, and male rats have been found to have an earlier onset of cancer compared to female animals *(9)*. Thus, the consistency of the experi-

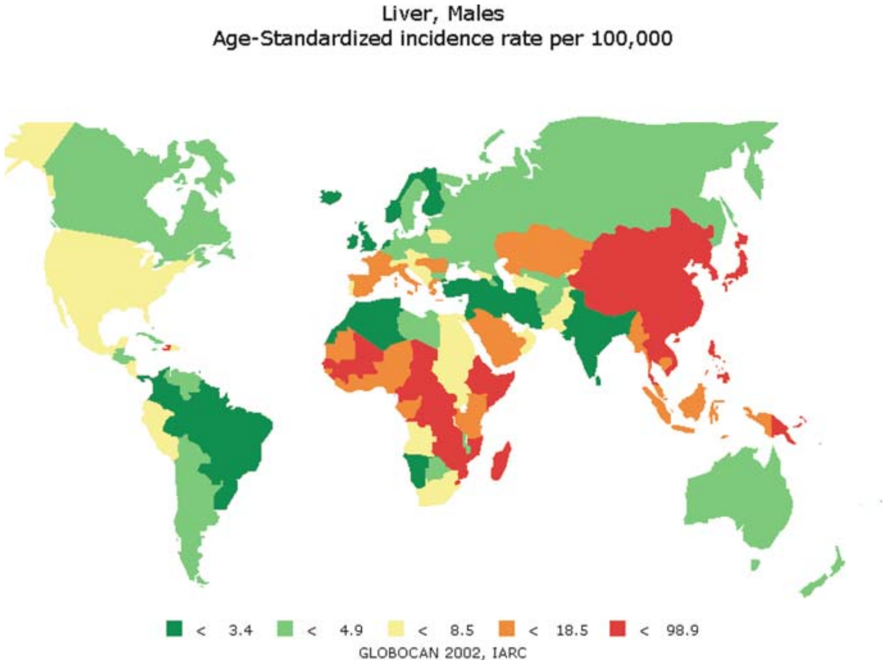

Fig. 1. Age-standardized incidence of liver cancer in men worldwide *(8)*.

mental animal and human data points to the important role that environmental exposures play in gender differences in HCC risk.

This chapter will review the significant data that link exposures to specific environmental carcinogens and the development of HCC in many parts of the world. These epidemiologic studies have been made possible by devising biomarkers reflective of exposure and risk. The translation of these basic science findings to an understanding of the etiology of HCC has also provided guidance for the development of preventive interventions in high-risk populations. We will review a number of these major investigations to provide an overview of this very active field of research.

2. MOLECULAR BIOMARKERS FOR ENVIRONMENTAL CARCINOGENS

Molecular biomarkers are typically used as indicators of exposure, effect, or susceptibility for both individuals and communities. A biomarker of exposure refers to measurement of the specific compound of interest, its metabolite(s), or its specific interactive products in a body compartment or fluid, which indicates the presence and magnitude of current and past exposure. A biomarker of effect indicates the presence and magnitude of a biological response from exposure to an environmental agent. Such a biomarker may be an endogenous component, a measure of the functional capacity of the system, or an altered state recognized as impairment or disease. A biomarker of susceptibility is an indicator or a metric of an inherent or acquired ability of an individual to respond to the challenge of exposure to a specific toxicant. Such a biomarker may be the unusual presence or absence of an endogenous component, or an abnormal functional response to an administered challenge *(10)*. Measures of these biomarkers through molecular epidemiology studies have great utility in addressing the relationships between exposure to environmental agents and development of clinical diseases, and in identifying those individuals at high risk for the disease *(11, 12)*. These data also help to inform the risk assessment process, where the effectiveness of regulations can be tested against biological measurements of exposure and effect.

The validation of any biomarker–effect link requires parallel experimental and human studies *(13)*. Following the development of a hypothesis of an exposure disease linkage, there is the need to devise the analytical methodology necessary to measure these biological markers in human and experimental samples. Conceptually, an appropriate animal model is often used to determine the associative or causal role of the biomarker in the disease or effect pathway, and to establish relations between dose and response. The putative biomarker can be validated in pilot human studies, where sensitivity, specificity, accuracy, and reliability parameters can be established. Data obtained in these studies can then be extended to assess intra- or interindividual variability, background levels, relationship of the biomarker to external dose or to disease status, as well as feasibility for use in larger population-based studies. To fully interpret the information that the biomarker provides, prospective epidemiological studies may be necessary to demonstrate the role that the biomarker plays in the overall pathogenesis of the disease or effect. Finally, these biomarkers can be translated as intermediate endpoints in interventions in both experimental models and high-risk human populations to optimize agent selection, dose, and schedule and other parameter influencing efficacy.

3. ENVIRONMENTAL ETIOLOGY OF HCC

As described above, HCC is among the leading causes of cancer death in most parts of the economically developing world. The unequal distribution of this disease is depicted by the map in Fig. 1 based upon the IARC cancer database *(8)*. Since the level of HCC is also coincident with regions where aflatoxin exposure is high, many efforts starting over 40 years ago examined this possible association. These initial studies were hindered by the lack of adequate data on aflatoxin intake, excretion, and metabolism in people, the underlying susceptibility factors such as diet and viral exposure, as well as by the incomplete statistics on worldwide cancer morbidity and mortality. Despite these deficiencies, early studies did provide data illustrating that increasing HCC rates corresponded to increasing levels of dietary aflatoxin exposure *(14)*. The commodities most often found to be contaminated by aflatoxin were common human food staples including: peanuts, cottonseed, corn, and rice *(15)*. The requirements for aflatoxin production are relatively non-specific since molds can produce these toxins on almost any foodstuff and the final levels in the grain product can vary from microgram to tens of milligrams *(16)*. Indeed, in a recent case of aflatoxin-related deaths in rural villages in Kenya, daily exposures were estimated to be over 50 mg *(17)*. Because contamination of foodstuffs is so heterogeneous, the measurement of human exposure to aflatoxin by sampling foodstuffs or by dietary questionnaires is extremely imprecise. The development and validation of specific aflatoxin biomarkers represents a significant advance for accurate assessment of exposure in biofluids such as urine and blood.

Concurrent with the early aflatoxin research were a series of studies describing a role for the hepatitis B virus (HBV) in HCC pathogenesis. A number of investigations found that chronic carriers of HBV, as indicated by sequential hepatitis B surface antigen (HBsAg) positivity at 6-month intervals, were at increased risk of developing HCC *(18)*. Further, the age of initial infection was directly related to development of the chronic carrier state and subsequent risk for HCC. Approximately 90% of HBV infections acquired in infancy or early childhood become chronic, whereas only 10% of infections acquired in adulthood become chronic, and less than 50% of chronic carriers progress to HCC *(5, 19–21)*. Finally, the global burden of HBV infection varies geographically and China, Southeast Asia, and sub-Saharan Africa have some of the highest rates of chronic HBV infection in the world, with prevalence of over 10% *(22)*. The public health significance of HBV as a risk factor for HCC is staggering with the consideration that there are over 400 million viral carriers and between 10 and 25% of these individuals are likely to develop HCC *(5, 23, 24)*. The biology, mode of transmission, and epidemiology of this viral infection continues to be actively investigated and has been recently reviewed *(22, 23, 25)*.

To date, the overwhelmingly significant etiological factors associated with development of HCC in the economically developing world are infection in early life with hepatitis B virus (HBV) and lifetime exposure to high levels of aflatoxin B_1 (AFB_1) in the diet *(26, 27)*. Indeed, the multiplicative interaction between HBV and AFB_1 has been documented in two separate cohorts at high risk for HCC *(28–30)*. Over the past 20 years, an appreciation for the role of the hepatitis C virus (HCV) has also emerged. HCV is contributing to HCC being the most rapidly rising solid tumor in the United States and Japan *(31)*. Detailed knowledge of the etiology of HCC has spurred many mechanistic studies to understand the pathogenesis of this nearly always fatal disease *(2, 26, 32)*.

A number of other environmental exposures have been epidemiologically associated with HCC *(33)*. Vinyl chloride exposure in occupational settings has been associated with the development of HCC in workers and there are now the classic studies associating vinyl chloride exposure with angiosarcomas in the liver *(34–36)*. Recently, studies have found a multiplicative interaction between vinyl chloride exposure in the workplace and alcohol consumption in the enhancement of HCC *(37)*. Finally, a synergistic interaction between vinyl chloride workplace exposure and HBV status has been reported in a cohort in Taiwan *(38)*.

Alcohol is a recognized human carcinogen and has been causally linked to HCC. Alcoholic cirrhosis and heavy alcohol use have been repeatedly associated with an increase in HCC risk *(39)*. However, it is unclear if alcohol use in the absence of cirrhosis influences HCC development *(40)*. Several studies have demonstrated an increased risk of HCC up to 5-fold with consumption of more than 80 g of alcohol per day or approximately 6–7 drinks per day *(39)*. The risk of HCC ranges from borderline significant to doubled with chronic alcohol consumption of less than 80 g/day *(39)*. A synergism between alcohol and HBV and HCV infections has also been described *(39, 41)*. In addition to the association of alcohol and HCC, in economically developed countries the dramatic rise in obesity and nonalcoholic fatty liver disease has also been related to increased HCC *(42–44)*.

Cigarette smoke is a recognized human carcinogen, however, a causal role in HCC is unclear *(45)*. A recent hospital-based case–control study in Italy found no independent effect for tobacco and HCC risk *(46)*. However, a composite analysis of tobacco exposure and cancer risk consistently shows a risk for liver cancer and smoking *(47)*. Finally, the role of hormones in the development of HCC is unclear; however, in some studies, an increased risk of HCC was observed among users of oral contraceptives *(48–50)*. Collectively, these hormonal-related increases in HCC are only seen in low incident countries where exposures to the other major risk factors for this cancer are rare.

4. METHODS FOR BIOMARKER MEASUREMENT

In the case of AFB_1, the measurement of the DNA and protein adducts were of major interest because they are direct products of (or surrogate markers for) damage to a critical cellular macromolecular target. The chemical structures and metabolic pathways leading to the formation of the major aflatoxin macromolecular DNA and protein adducts were known (Fig. 2) *(51, 52)*. The finding that the major aflatoxin–nucleic acid adduct AFB_1–N^7-Gua was excreted exclusively in urine of exposed rats spurred interest in using this metabolite as a biomarker of both exposure and risk. This

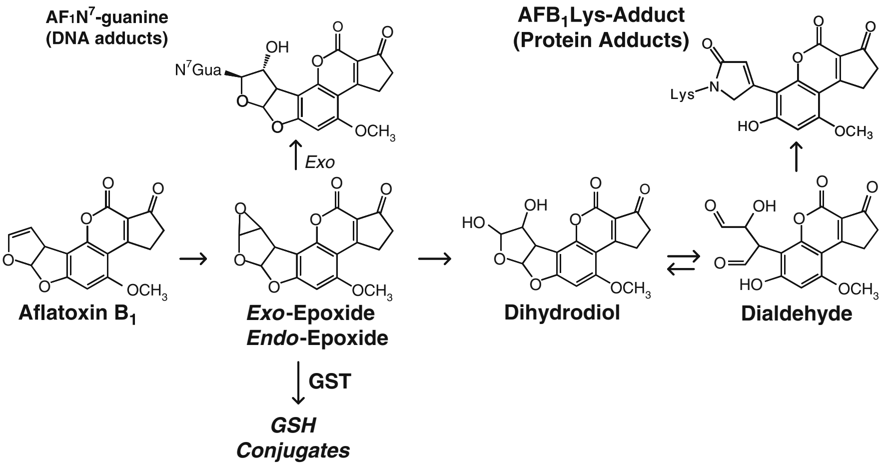

Fig. 2. Structures of aflatoxin biomarkers.

adjunct, however, has a short half-life in the body (~8 h) *(53)*. The serum aflatoxin–albumin adduct was also examined as a biomarker since the longer half-life of albumin (~3 weeks) integrates exposures over longer time periods. Studies in experimental models found that the formation of aflatoxin–DNA adducts in liver, excretion of the urinary aflatoxin–nucleic acid adduct, and formation of the serum albumin adduct were highly correlated *(54)*.

Many different analytical methods were available for quantitation of chemical adducts in biological samples *(55–57)*. Each methodology has unique specificity and sensitivity and, depending on the application, the user can choose which is most appropriate. For example, to measure a single aflatoxin metabolite, a chromatographic method can resolve mixtures of aflatoxins into individual compounds, providing that the extraction procedure does not introduce large amounts of interfering chemicals. Antibody-based methods were often more sensitive than chromatography, but immunoassays are less selective because the antibody may cross-react with multiple metabolites. A recent inter-laboratory collaboration used identical serum sample sets to analyze for aflatoxin–albumin adducts by ELISA, high-performance liquid chromatography (HPLC) with fluorescence detection (HPLC-f), and HPLC with isotope-dilution mass spectrometry (IDMS). Overall, this study showed an excellent correlation between these three independent methodologies conducted in different laboratories *(58)*.

An immunoaffinity cleanup/HPLC procedure was developed to isolate and measure aflatoxin metabolites in biological samples *(59–61)*. With this approach, we performed initial validation studies for the dose-dependent excretion of urinary aflatoxin biomarkers in rats after a single exposure to AFB_1 *(62)*. A linear relationship was found between AFB_1 dose and excretion of the AFB–N^7-Gua adduct in urine over the initial 24 h period of exposure. In contrast, excretion of other oxidative metabolites, such as AFP_1 showed no linear association with dose. Subsequent studies in rodents that assessed the formation of aflatoxin macromolecular adducts after chronic administration also supported the use of DNA and protein adducts as molecular measures of exposure *(63, 64)*. Recent studies using isotope-dilution mass spectrometry with liquid chromatography separation have demonstrated an increase in sensitivity of at least 1,000-fold over technologies used for the detection of aflatoxin biomarkers 15 years ago *(65–67)*. Further, repeated analysis of serum collected in 1983 from aflatoxin-exposed people has demonstrated that the aflatoxin–lysine adduct in albumin is stable under a range of temperature storage conditions *(68)*.

An area of considerable importance, that has received far less attention than it should, has been in the area of internal standard development. All quantitative measurements require the use of an internal standard to account for sample to sample variations in the analyte recoveries. In the case of mass spectrometry, internal standards generally employ an isotopically labeled material that is identical to the chemical being measured. Obtaining such isotopically labeled materials requires chemical synthesis, if they are not commercially available, and has impeded the application of internal standards in many studies. In the case of immunoassays, internal standards pose a different challenge since the addition of an internal standard that is recognized by an antibody results in a positive value contribution. The dynamic range is usually less than 100 in immunoassays, and therefore great care must be taken to spike a sample with an internal standard so one can obtain a valid result. In contrast, most chromatographic methods result in dynamic ranges of analyses that can be over a 10,000-fold range of levels. The mass spectrometry methods are not only applicable for the quantitation of small molecules such as aflatoxin, but it has also been extended for use to measure mutations in DNA fragments found circulating in plasma that are mechanistically linked to the etiopathogenesis of HCC, such as p53 *(69–72)*.

5. VALIDATION OF BIOMARKERS OF ENVIRONMENTAL CARCINOGENS

In the early 1980s studies to identify effective chemoprevention strategies for aflatoxin carcinogenesis was initiated. The hypothesis was that reduction of aflatoxin–DNA adduct levels by chemopreventive agents would be mechanistically related to and therefore predictive of cancer preventive efficacy. Preliminary data with a variety of established chemopreventive agents demonstrated that after a single dose of aflatoxin, levels of DNA adducts were reduced *(73)*. A more comprehensive study using multiple doses of aflatoxin and the chemopreventive agent, ethoxyquin, was carried out to examine the relationships between levels and rates of DNA adduct formation and removal and hepatic tumorigenesis in rats. At 3 months after aflatoxin treatment, it was observed that co-treatment with ethoxyquin had reduced both area and volume of liver occupied by presumptive preneoplastic foci by >95%. This same protocol also dramatically reduced binding of AFB_1 to hepatic DNA, from 90% initially to 70% over the course of a 2-week carcinogen-dosing period. Intriguingly, no differences in residual DNA adduct burden, however, were discernible several months after dosing despite the profound reduction in tumor burden.

The experiment was then repeated with several different chemopreventive agents and in all cases aflatoxin-derived DNA and protein adducts were reduced; however, even under optimal conditions, the reduction in the macromolecular adducts always under-represented the magnitude of the diminution in tumor burden *(74, 75)*. These macromolecular adducts can track with disease outcome on a population basis, but in the multistage process of cancer the absolute level of adduct provides only a necessary but insufficient measure of tumor formation.

Experimental validation of the role of human HBV in HCC etiopathogenesis has been compromised by the very restricted nature of the number of species that can become infected with this virus. The chimpanzee and tree shrew can be infected by human HBV but neither has proven to be a cost-effective model for extensive investigation, while the woodchuck and duck can be infected with similar yet distinct HBV strains *(76–78)*. Transgenic mouse models have also been developed that generate a 100% probability of developing HCC *(79)*. These transgenic mice have been used to explore the interaction of the HBV transgene with AFB_1 *(80)*. Collectively, these models are extremely valuable for the study of the underlying molecular pathways in the virally induced cancers but they have to date been of limited value in recapitulating the more complex etiology of human HCC.

Using the chemopreventive agent oltipraz, Roebuck et al. *(74)* established correlations between reductions in levels of AFB_1–N^7-Gua excreted in urine and incidence of HCC in aflatoxin-exposed rats. Overall, reduction in biomarker levels reflected protection against carcinogenesis, but these studies did not address the quantitative relationship between biomarker levels and individual risk. Thus, in a follow-up study, rats dosed with AFB_1 daily for 5 weeks were randomized into three groups: no intervention; delayed-transient intervention with oltipraz during weeks 2 and 3 of exposure; persistent intervention with oltipraz for all 5 weeks of dosing *(81)*. Serial blood samples were collected from each animal at weekly intervals throughout aflatoxin exposure for measurement of aflatoxin–albumin adducts. The integrated level of aflatoxin–albumin adducts over the exposure period decreased to 20% and 39% in the delayed-transient and persistent oltipraz intervention groups, respectively, as compared with no intervention. Similarly, the total incidence of HCC dropped significantly from 83 to 60% and 48% in these groups. Overall, there was a significant association between integrated biomarker level and risk of HCC. When the predictive value of aflatoxin–serum albumin adducts was assessed within treatment groups, however, there was no association between integrated biomarker levels and risk of HCC. These data clearly demonstrated that levels of the aflatoxin–albumin adducts could predict population-based changes in disease risk, but had no power to identify individuals destined to develop HCC. Because of the multistage process of carcinogenesis, in order to determine individual risk of disease, a panel of biomarkers reflecting different stages will be required.

6. BIOMARKERS IN HUMAN INVESTIGATIONS

Extensive cross-sectional epidemiologic studies have been conducted in high-risk groups for HCC. The HBV biomarkers were developed and validated using the HBsAg biomarker. This work directly led to the research that resulted in a vaccine effective against HBV. Indeed, this vaccine has been reported to reduce HCC in a cohort of young children in Taiwan *(82)*. Further the serology of HBV has been extensively described and developed *(25)*. The work on AFB_1 exposures and its role in HCC etiology has taken a far longer time period to come to fruition. Initial studies in the Philippines *(83)* demonstrated that an oxidative metabolite of aflatoxin could be measured in urine and thus had potential to serve as an internal dose marker. Subsequent work conducted in the People's Republic of China and The Gambia, West Africa, areas with high incidences of HCC, determined that the levels of urinary aflatoxin biomarkers showed dose-dependent relationships with aflatoxin intake. Gan et al. *(84)* and Wild et al. *(85)* also monitored levels of aflatoxin serum albumin adducts and observed a highly significant association between intake of aflatoxin and level of adduct. Many of the aflatoxin studies used different analytical methods and therefore the quantitative comparison of different data sets has been extremely problematic. However, a recent study compared methods of ELISA and mass spectrometry (MS) and found high correlation between these two methods ($r = 0.856$, $p < 0.0001$) *(66)*.

Biomarker development in HCC has been further advanced by the molecular biological studies on the TP53 tumor suppressor gene, the most common mutated gene detected in human cancer *(86, 87)*. Many studies of *p53* mutations in HCC occurring in populations exposed to high levels of dietary aflatoxin have found high frequencies of guanine to thymine transversions, with clustering at codon 249 *(88, 89)*. In contrast, no mutations at codon 249 were found in *p53* in HCC from Japan and other areas where there was little exposure to aflatoxin *(90, 91)*. The occurrence of this specific mutation has been mechanistically associated with AFB_1 exposure in experimental models including bacteria *(92)* and through demonstration that aflatoxin-8,9-epoxide could bind to codon 249 of *p53* in a DNA plasmid in vitro *(93)*. Mutational analysis of the *p53* gene in human HepG2 cells and hepatocytes exposed to AFB_1 found preferential induction of the transversion of guanine to thymine in the third position of codon 249 *(94, 95 96, 97)*. In summary, studies of the prevalence of codon 249 mutations in HCC cases from patients in areas of high or low exposure to aflatoxin suggest that a G–T transition at the third base is associated with aflatoxin exposure and in vitro data would seem to support this hypothesis.

Although useful, cross-sectional epidemiological studies have the least power to relate an exposure to disease outcome since these studies only provide a view during a short time frame. Data from the cross-sectional aflatoxin biomarker studies demonstrated short-term dose–response effects for a number of the aflatoxin metabolites, including the major nucleic acid adduct, serum albumin adduct, and AFM_1 This information could then be used in follow-up studies to test a number of hypotheses about risk to individuals having high exposures, the efficacy of exposure remediation, and interventions and mechanisms underlying susceptibility.

Longitudinal studies are extremely important in the development and validation process for biomarkers. These investigations permit an understanding of the stability in storage and the tracking potential of each biomarker, which are essential for the evaluation of the predictive power of the biomarker. While long-term stability of many of the HBV markers have been well-established *(98)*, we needed to know whether the aflatoxin metabolites were stable over the long term. The stability of aflatoxin biomarkers was monitored by supplementing urine samples with aflatoxins at the time of collection and then analyzing repeated samples over the course of 8 years. Similarly, aflatoxin–albumin adducts, as described above, in human sera were found to be stable for at least 15 years when stored at –20°C *(68)*. Therefore, at least for some of the aflatoxin biomarkers, degradation over time was not a major problem; however, similar studies are required for all chemical-specific biomarkers.

An objective in development of any biomarker is to use them as predictors of past and future exposure status in people. This concept is embodied in the principle of tracking, which is an index of how well an individual's biomarker remains positioned in a rank order relative to other individuals in a group over time. Tracking within a group of individuals is expressed by the intraclass correlation coefficient. When the intraclass correlation coefficient is 1.0, a person's relative position, independent of exposure, within the group does not change over time. If the intraclass correlation coefficient is 0.0, there is random positioning of the individual's biomarker level relative to the others in the group throughout the time period. The tracking concept is central to interpreting data related to exposure and biomarker levels and requires acquisition of repeated samples from subjects. Unfortunately, data on the temporal patterns of formation and persistence of aflatoxin macromolecular adducts in human samples are very limited. Obviously, chemical-specific biomarkers measured in cross-sectional studies cannot provide information on the predictive value or tracking of an individual's marker level over time. In contrast to the aflatoxin situation, the HBV biomarker tracking has been well characterized and forms the basis for defining chronic infection status *(98)*.

Tracking is important in assessing exposure and this information is essential in the design of intervention studies. In all these situations it is critical to know how many biomarker samples are required and when they should be obtained. For example, if exposure remains constant and the tracking value for a marker changes over time, it might be assumed that the change in tracking is due to a biological process, such as an alteration in the balance of metabolic pathways responsible for adduct formation. On the other hand, lack of tracking can be attributable to great variance in exposure. Therefore, to determine unequivocally the contributions of intra- and interindividual variations to biomarker levels, experiments must assess tracking over time.

Many published case–control studies have examined the relation of aflatoxin exposure and HCC. Compared with cohort studies, case–control studies are both cost- and time-effective. Unfortunately, case–control studies are often initiated long after exposure has occurred and it cannot be assumed that the exposure has not appreciably changed over time. Also, such studies involve assumptions in the selection of controls, including that the disease state does not alter metabolism of aflatoxin. Thus, matching of cases and controls in a specific biomarker study is much more difficult than in a case–control study involving genetic markers.

One of the first case–control studies compared the dietary intake of aflatoxin in cases of HCC in the Philippines with intake in age- and sex-matched controls. Bulatao-Jayme et al. *(99)* found that the mean aflatoxin exposure per day in cases of HCC was 4.5 times higher than in the controls; however, alcohol consumption was a confounder in this study that may have enhanced this effect. In the Guangxi Autonomous Region of China *(100, 101)* the interaction between HBV infection and dietary aflatoxin exposure dichotomized for heavy and light contamination was examined. Those individuals who were positive for HBsAg and had heavy aflatoxin exposure had an incidence of HCC 10-fold higher than did people living in areas with light aflatoxin contamination *(100)*. In a case–control study in Taiwan, two biomarkers, aflatoxin–albumin adducts and aflatoxin–DNA adducts in liver tissue samples, were measured *(102)*. The proportion of subjects with a detectable level of aflatoxins–albumin adducts was higher for cases of HCC than for matched controls (odds ratio 1.5). There was also a statistically significant association between detectable level of aflatoxin–albumin adduct and risk of HCC among men younger than 52 years old (multivariant adjusted odds ratio 5.3). Although a number of negative case–control studies of aflatoxin and HCC have been reported *(15)*, the overwhelming evidence from many investigations pointed to an etiological role for aflatoxin in human HCC.

Data obtained from cohort studies have the greatest power to determine a true relationship between an exposure and disease outcome because one starts with a healthy cohort, obtains biomarker samples, and then follows the cohort until significant numbers of cases are obtained. A nested study within the cohort can then be designed to match cases and controls. An advantage of this method is causation can be established (due to the longitudinal nature of cohort studies, there is no temporal ambiguity) and selection bias is minimized. A major disadvantage, however, is the time needed in follow-up (often years) to accrue the cases, especially for chronic diseases such as HCC. This disadvantage can be overcome in part by enrolling large numbers of people (often tens of thousands) to ensure case accrual at a reasonable rate.

To date two major cohort studies with aflatoxin biomarkers have demonstrated the important role of this carcinogen in the etiology of HCC. The first study, comprising more than 18,000 men in Shanghai, examined the interaction of HBV and aflatoxin biomarkers as independent and interactive risk factor for HCC. The nested case–control data revealed a statistically significant increase in the adjusted relative risk (RR) of 3.4 [95% CI: 1.1.–10.0] for those HCC cases where urinary aflatoxin biomarkers were detected. For HBsAg-positive people only the RR was 7 [95% CI: 2.2.–22.4], but for individuals with both urinary aflatoxins and positive HBsAg status the RR was 59 [95% CI: 16.6.–212.0] *(103, 104)*. These results strongly support a causal relationship between the presence of the chemical and viral-specific biomarkers and the risk of HCC.

Subsequent cohort studies in Taiwan have substantially confirmed the results from the Shanghai investigation. Wang et al. *(105)* examined HCC cases and controls nested within a cohort and found that in HBV-infected people there was an adjusted odds ratio of 2.8 [95% CI] for detectable compared with non-detectable aflatoxin–albumin adducts and 5.5 [95% CI] for high compared with low levels of aflatoxin metabolites in urine. In a follow-up study, there was a dose–response relationship between urinary AFM_1 levels and risk of HCC in chronic HBV carriers. Similar to the Shanghai study, the HCC risk associated with AFB_1 exposure was more striking among the HBV carriers with detectable AFB_1–N^7-gua in urine.

Many studies across the globe have explored the relationship between HBV infection and HCC and the risk estimates range from 3 to 30 in case–control studies and from 5 to 148 in cohort studies *(49)*. In the nested case–control study cited above the risk of HCC was 7.3 times higher among HBsAg-positive individuals compared to HBsAg-negative individuals, controlled for smoking and aflatoxin exposures *(29)*. A small hospital-based case–control study from northeast Thailand showed an adjusted odds ratio (OR) of 15.2 for the presence of HBsAg among HCC patients *(106)*. An adjusted OR of 13.5 was reported from a case–control study in The Gambia *(22)*. The risk of HCC among HBsAg positive individuals in Korea from a prospective cohort study of government workers was 24.3 among men and 54.4 among women, adjusted for age, smoking, alcohol use, and diabetes *(107)*. A similar prospective study from Taiwan found men positive for HBsAg were 223 times more likely to develop HCC than men with HBsAg negative *(20)*.

The contribution of HBV to the pathogenesis of liver cancer is multifactorial and is complicated by the identification of mutant variants in HBV that modulate the carcinogenesis process *(108, 109)*. The HBV genome encodes its essential genes with overlapping open-reading frames; therefore, a mutation in the HBV genome can alter the expression of multiple proteins. In many cases of HCC in China and Africa a double mutation in the HBV genome, an adenine to thymine transversion at nucleotide 1762 and a guanine to adenine transition at nucleotide 1764 ($1762^{T}/1764^{A}$), has been found in tumors *(110–112)*. This segment of the HBV genome contains an overlapping sequence for the base core promoter and the HBV X gene; therefore, the double mutation in codon 130 and 131 of the HBV X gene reported in human HCC is identical to the 1762 and 1764 nucleotide changes *(113)*. The increasing occurrence of these mutations have been also associated with the increasing severity of the HBV infection and cirrhosis *(111, 112)*. This acquired mutation following HBV integration into hepatocytes was originally characterized in HBV e antigen negative people *(114)*. The $1762^{T}/1764^{A}$ double mutation occurs more frequently in people infected with the genotype C strains of HBV, which is the most common genotype found in East Asian patients *(115–117)*. This double mutation tracks with an increased inflammatory response that becomes stronger as the progression of liver damage transits through chronic hepatitis and into a cirrhosis stage *(118)*. The underlying mechanism of the effects of HBV e antigen on the biology of inflammation and cirrhosis are still unclear, but there are substantial data that point to modulation of the immune surveillance system and immune tolerance in the presence and absence of this protein *(118–120)*. The $1762^{T}/1764^{A}$ double mutation has also been demonstrated to affect an increase in the rate of HBV genome synthesis in cellular models *(108, 109)*. In cellular studies the $1762^{T}/1764^{A}$ double mutation increased the replication of the viral genome 2-fold and in the case of some of the rarer triple mutations, an 8-fold increase in genome replication was found *(108, 120)*. Recent data have also shown that there is a sequential accumulation of these mutations in people during the course of the progression to cancer *(121)*.

7. INTERVENTION TRIALS USING AFLATOXIN BIOMARKERS

Clinical trials and other interventions are designed to translate findings from human and experimental investigations to public health prevention. Both primary (to reduce exposure) and secondary (to alter metabolism and deposition) interventions can use specific biomarkers as endpoints of efficacy. Such biomarkers can be applied to the preselection of exposed individuals for study cohorts, thereby reducing study size requirements. They can also serve as short-term modifiable endpoints *(122)*. In a primary prevention trial the goal is to reduce exposure to aflatoxins in the diet. Interventions can range from attempting to lower mold growth in harvested crops to using trapping agents that block the uptake of ingested aflatoxins. In secondary prevention trials one goal is to modulate the metabolism of ingested aflatoxin to enhance detoxification processes, thereby reducing formation of DNA adducts and enhancing elimination.

The use of aflatoxin biomarkers as efficacy endpoints in primary prevention trials in West Africa has been recently reported *(123)*. This study assesses postharvest measures to restrict aflatoxin contamination of groundnut crops. Six hundred people were monitored and in control villages mean aflatoxin–albumin concentration increased postharvest (from 5.5 pg/mg [95% CI: 4.7–6.1] immediately after harvest to 18.7 pg/mg [17.0–20.6] 5 months later). By contrast, mean aflatoxin–albumin concentration in intervention villages after 5 months of groundnut storage was much the same as that immediately postharvest (7.2 pg/mg [6.2–8.4] vs. 8.0 pg/mg [7.0–9.2]). At 5 months, mean adduct concentration in intervention villages was less than 50% of that in control villages (8.0 pg/mg [7.2–9.2] vs. 18.7 pg/mg [17.0–20.6], $p < 0.0001$). Thus, primary prevention may be an effective means to reduce HCC burden, especially in areas where single foodstuffs such as groundnuts are major components of the diet.

Aflatoxin biomarkers were also used as intermediate biomarkers in a Phase IIa chemoprevention trial of oltipraz in Qidong, PRC *(124–126)*. This was a placebo-controlled, double-masked study in which participants were randomized to receive placebo or 125 mg oltipraz daily or 500 mg oltipraz weekly. Urinary AFM_1 levels were reduced by 51% compared with the placebo group in persons receiving the 500 mg weekly dose. No significant differences were seen in urinary AFM_1 levels in the 125 mg group compared with placebo. This effect at the higher dose was thought to be due to inhibition of cytochrome P450 1A2 activity. Median levels of AFB_1–mercapturic acid (a glutathione conjugate derivative) were elevated 2.6-fold in the 125 mg group, but were unchanged in the 500 mg group. Increased AFB_1–mercapturic acid reflects induction of aflatoxin conjugation through the actions of glutathione S-transferases. The apparent lack of induction in the 500 mg group probably reflects masking due to diminished substrate formation for conjugation through the inhibition of CYPlA2 seen in this group.

This strategy was extended to chlorophyllin, an anticarcinogen in experimental models when given in large molar excess relative to the carcinogen at or around the time of carcinogen exposure. Chlorophyllin cuts by forming molecular complexes with carcinogens such as aflatoxin in the gastrointestinal tract, thereby blocking bioavailability. One hundred eighty healthy adults from Qidong were randomly assigned to ingest 100 mg chlorophyllin or a placebo three times a day for 4 months. The primary endpoint was modulation of levels of aflatoxin–N^7-guanine adducts in urine samples collected 3 months into the intervention measured using sequential immunoaffinity chromatography and liquid chromatography–electrospray mass spectrometry. Chlorophyllin consumption at each meal led to an overall 55% reduction in median urinary levels of this aflatoxin biomarker compared to those taking placebo *(127)*. Recently, we tested whether drinking hot water infusions of 3-day-old broccoli sprouts, containing defined concentrations of glucosinolates as a stable precursor of the anticarcinogen sulforaphane, could alter the disposition of aflatoxin. Sulforaphane, like oltipraz, acts to increase expression of aflatoxin detoxication enzymes in the liver and other tissues. Two hundred healthy adults drank infusions containing either 400 or < 3 μmol glucoraphanin nightly for 2 weeks. Urinary levels of AFB_1–N^7-Gua were not different between the two intervention arms. However, measurement of urinary levels of dithiocarbamates (sulforaphane metabolites) indicated striking interindividual differences in bioavailability. Presumptively, there were individual differences in the rates of hydrolysis of glucoraphanin to sulforaphane by the intestinal microflora of the study participants. Nonetheless, an inverse association was observed for excretion of dithiocarbamates and aflatoxin–DNA adducts in individuals receiving broccoli sprout glucosinolates *(128)*.

8. DNA MUTATIONS MEASURED IN HUMAN PLASMA AND HCC

The development and validation of biomarkers for early detection of disease or for the identification of high-risk individuals is a major translational effort in cancer research. α-Fetoprotein is widely used as a HCC diagnostic marker in high-risk areas because of its ease of use and low cost. *(129)*

However, this marker suffers from low specificity due to its occurrence in diseases other than liver cancer. Moreover, no survival advantage is seen in populations when α-fetoprotein is used in large-scale screening *(130)*. Such inadequacies have contributed to the need to identify other molecular biomarkers that are possibly more mechanistically associated with HCC development, including hypermethylation of the p16 gene, p15 gene, GSTP1 promoter regions, and codon 249 mutations in the p53 gene *(131–134)*. Results from investigations of p16, p15, GSTP1 promoter hypermethylation, and p53 mutations indicate that these markers are prevalent in HCC, but there is as of yet limited information on the temporality of these genetic changes prior to clinical diagnosis.

Several studies have now demonstrated that DNA isolated from serum and plasma of cancer patients contains the same genetic aberrations as DNA isolated from an individual's tumor *(70, 135, 136)*. The process by which tumor DNA is released into circulating blood is unclear but may result from accelerated necrosis, apoptosis, or other processes *(137)*. While the detection of specific p53 mutations in liver tumors has provided insight into the etiology of certain liver cancers, the application of these specific mutations to the early detection of cancer offers great promise for prevention *(138)*. In a seminal report, Kirk et al. *(139)* reported the detection of codon 249 p53 mutations in the plasma of liver tumor patients from The Gambia; however, the mutational status of the tumors was not known. These authors also reported a small number of cirrhosis patients having this mutation and given the strong relation between cirrhosis and future development of HCC, raised the possibility of this mutation being an early detection marker. Jackson et al. *(140)* used short oligonucleotide mass analysis (SOMA) in lieu of DNA sequencing for analysis of specific p53 mutations in HCC samples. Analysis of 20 plasma and tumor pairs showed 11 tumors containing the specific mutation, 6 of the paired plasma samples exhibited the same mutation.

The temporality of the detection of this mutation in plasma before and after the clinical diagnosis of HCC was facilitated by the availability of longitudinally collected plasma samples from a cohort of 1,638 high-risk individuals in Qidong, PRC, that have been followed since 1992 *(141)*. The results showed that in samples collected prior to liver cancer diagnosis, 21.7% of the plasma samples had detectable levels of the codon 249 mutation. The persistence of this prediagnosis marker was borderline statistically significant. The codon 249 mutation in p53 was detected in 44.6% of all plasma samples following the diagnosis of HCC. Collectively these data suggest that nearly one half of the potential patients with this marker can be detected at least 1 year and in 1 case 5 years prior to diagnosis.

Using a novel internal standard plasmid, plasma concentrations of p53 codon 249-mutated DNA were quantified by SOMA in 89 hepatocellular carcinoma cases, 42 cirrhotic patients, and 131 nonliver diseased control subjects, all from highly aflatoxin-exposed regions of The Gambia *(72)*. The hepatocellular carcinoma cases had higher median plasma concentrations of the p53 mutation (2,800 copies/mL; interquartile range: 500–11,000) compared with either cirrhotic (500 copies/mL; interquartile range: 500–2,600) or control subjects (500 copies/mL; interquartile range: 500–2,000). Levels of >10,000 copies of p 53 codon 249 mutation/mL plasma were also significantly associated with the diagnosis of HCC (odds ratio, 15; 95% confidence interval, 1.6–140) when compared with cirrhotic patients. Potential applications for the quantification of this alteration of DNA in plasma include estimation of long-term, cumulative aflatoxin exposure and selection of appropriate high-risk individuals for targeted intervention.

In many cases of HCC in China and Africa a double mutation in the HBV genome, an adenine to thymine transversion at nucleotide 1762 and a guanine to adenine transition at nucleotide 1764 (1762T/1764A), has been found in tumors *(142, 143)*. Kuang et al. *(144)* examined, with mass spectrometry, the temporality of an HBV 1762T/1764A double mutation in plasma and tumors. Initial studies found 52 of 70 (74.3%) tumors from Qidong, PRC contained this HBV mutation. Paired plasma samples were available for six of the tumor specimens; four tumors had the HBV 1762T/1764A mutation while three of the paired plasma samples were also positive. The potential predictive value of this biomarker was explored using stored plasma samples from a study of 120 residents of Qidong who had been monitored for aflatoxin exposure and HBV infection. After 10 years passive follow–up, there were six cases of major liver disease and all had detectable levels of the HBV 1762T/1764A mutation up to 8 years prior to diagnosis. Finally, 15 liver cancers were selected from a prospective cohort of 1,638 high-risk individuals in Qidong and the HBV 1762T/1764A mutation was detected in 8 of the 15 cases prior to cancer. The persistence of detection of this mutation was statistically significant. We have therefore found that a prediagnosis biomarker of specific HBV mutations can be measured in plasma and suggest this marker for use as an intermediate endpoint in prevention and intervention trials.

9. SUMMARY

HCC is a slowly developing process involving progressive genetic insults and their resulting genomic changes *(145, 146)*. HCC may not become evident until over 30 years after chronic infection with HBV, HCV, and/or aflatoxin exposure. Chronic hepatitis and cirrhosis may only develop 5 years before HCC is evident and globally, 70–75% of all HCC is accompanied by cirrhosis *(110, 145)*. This genomic heterogeneity may be a reflection of the different etiologies of HCC and their effect upon the molecular regulation of hepatocytes *(146)*. Over the past 25 years, the development and application of molecular biomarkers reflecting events from exposure to manifestation of clinical diseases has rapidly expanded our knowledge of the mechanisms of HCC pathogenesis. These biomarkers will have increasing potential for early detection, treatment, and prevention.

The molecular epidemiology investigations of aflatoxin, HBV, and HCC probably represent one of the most extensive data sets in the field of environmental carcinogenesis and this work may serve as a template for future studies of the role of other environmental agents in human diseases with chronic, multifactorial etiologies (Fig. 3). The development of these biomarkers has been based upon the knowledge of the biochemistry and toxicology of aflatoxins gleaned from both experimental and human studies. These biomarkers have subsequently been utilized in experimental models to provide data on the modulation of these markers under different situations of disease risk. This systematic approach provides encouragement for design and successful implementation of preventive interventions.

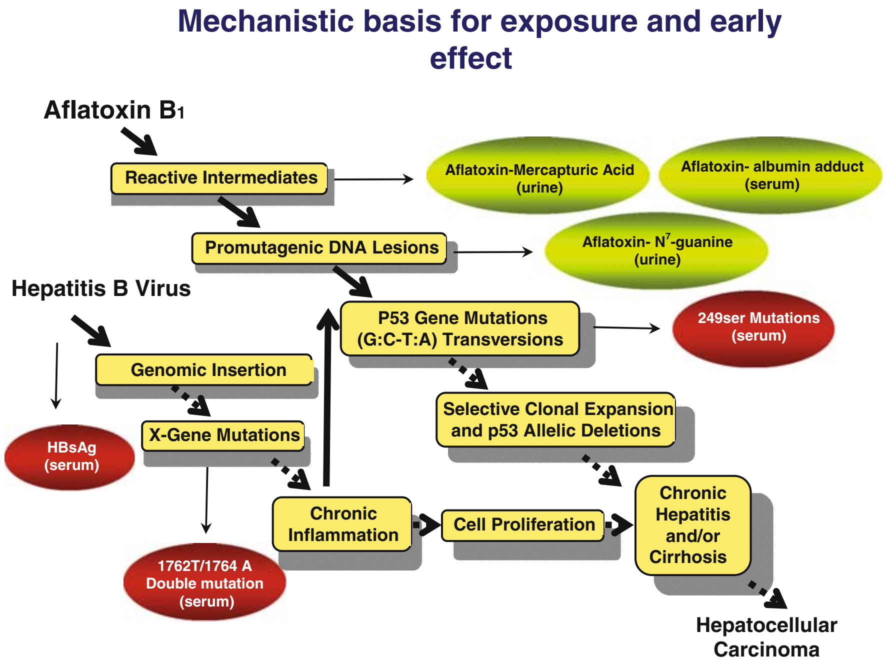

Fig. 3. Mechanistic-based biomarkers of aflatoxin and HBV.

ACKNOWLEDGMENTS

This work was supported in part by grants P01 ES006052, R01 CA39416, and P30 ES003819 from the USPHS.

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