Prevention of Liver Cancer in Qidong, China: Lessons from Aflatoxin Biomarker Studies

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Abstract Primary liver cancer has been the leading cause of cancer death in Qidong, China, with about 800 deaths annually in this region of 1.1 million residents. Epidemiological studies have highlighted the importance of infection with hepatitis B virus (HBV) and dietary exposure to hepatocarcinogenic aflatoxins as key, interactive determinants of risk in Qidong and other endemic areas. Identification of these risk factors was enabled through the development of biomarkers allowing for measures of their prevalence in case-control and other study cohorts. Vaccination against HBV began in pilot studies in the 1980s in Qidong but did not become universal for newborns until earlier this decade. Despite minimal impact on cancer mortality to date, vaccination programs are poised to blunt the development of liver diseases including cancer in this region over the next generations. Strategies for reducing aflatoxin exposure are also required, especially for those already infected with HBV. We have conducted a series of proof-of-principle clinical trials in which drugs, dietary supplements and foods have been used to alter the metabolism and elimination of aflatoxins following unavoidable exposures. Using aflatoxin biomarkers as intermediate endpoints, the efficacy of these agents (oltipraz, chlorophyllin, broccoli sprout beverages) has been demonstrated, highlighting roles for frugal approaches to chemoprevention against environmental carcinogenesis. Remarkably, recent evidence garnered from retrospective analysis of archived serum samples from the past quarter century demonstrates a 40-fold drop in aflatoxin exposure in Qidong, likely driven by changes in the primary dietary staple from maize to rice. Thus, primary prevention, evoked by changing economic and agricultural policies in the 1980s, heralds the unanticipated promise of elimination of liver cancer from this endemic region in a fore-shortened timeframe.

Key words aflatoxin-N7-guanine; aflatoxin-albumin adduct; primary liver cancer; chemoprevention

1 Introduction

Primary liver cancer (PLC) is the third leading cause of cancer mortality worldwide and results in nearly 700 000 deaths annually; about one-half occur in China [1]. PLC, the leading cause of cancer death in Qidong in eastern Jiangsu province until the recent, countrywide emergence of the lung cancer epidemic, still accounts for up to 10% of all adult deaths in some of the rural townships [2,3]. Crude mortality rates from PLC have risen over the past decades in Qidong and survival after diagnosis remains poor [4]. Chronic infection with hepatitis B virus (HBV) and exposure to aflatoxins in the diet contribute to the extraordinarily high risk of PLC in Qidong; these two factors likely are responsible for 90% or more of the cases of PLC in this endemic region.

Several nested case-control studies in Shanghai, Qidong and Taiwan have demonstrated an interaction between HBV and aflatoxins for risk of PLC [5−7]. From

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a public health perspective, these findings suggest that HBV vaccination programs and efforts to reduce aflatoxin exposures could have major impact on mortality from this disease. Indeed, a pilot program for vaccination of newborns against HBV infection began in some Qidong townships in the 1980s [8], however, comprehensive access for all newborns did not occur until the early part of this century when the central government made both the vaccine and its administration free of cost. Infection with HBV typically occurs in the perinatal period. As a consequence an immunization program for total population protection would have to occur over several generations, provided that mutant strains of HBV do not arise.

Aflatoxins are potent hepatocarcinogens produced by fungi and have been important contaminants of the food supply in this area, particularly in maize, peanuts, soya sauce, and fermented soy beans. The extent of aflatoxin contamination in foods is a function of the ecology of molds and not completely preventable. Thus, elimination of aflatoxin exposures has been regarded as unfeasible in most of the high risk areas of the world because of the high economic costs associated with proper crop storage and handling or the challenges of making wholesale changes in the availability of dietary staples towards those with lower propensity for aflatoxin contamination. From this perspective, it has been our view that secondary prevention programs, such as chemoprevention, may be useful, especially in those already chronically infected with HBV [9]. Cancer chemoprevention entails the use of natural or synthetic agents to retard, block or even reverse the carcinogenic process. Indeed many agents have been described that inhibit aflatoxin carcinogenesis in animal models and major determinants of interspecies susceptibility to this carcinogen have been identified, further validating intervention targets [9,10]. In a few cases, promising agents have been used in clinical trials in the Qidong region, with demonstrable enhancement of aflatoxin detoxication and elimination. This perspective briefly reviews some of the most salient outcomes from these biomarker-based trials, but, with the benefit of several decades of hindsight and a treasure trove of archived serum and urine samples, also highlights the tremendous impact that primary prevention of aflatoxin exposure can have on PLC mortality, even under conditions of economic constraint.

2 Aflatoxin biomarkers

We and others have developed and validated a number of aflatoxin biomarkers for application in studies of PLC etiology and prevention [11]. As shown in Fig. 1, aflatoxin is metabolized by cytochrome P450s to a reactive epoxide (aflatoxin-8,9-epoxide) and other oxidation products, including aflatoxin M1. The epoxide can react further by interacting with DNA to form a promutagenic aflatoxin-N7-guanine adduct. This is an unstable adduct which rapidly undergoes depurination and excretion in the urine. The epoxide can also react with serum albumin to form long-lived lysine adducts. In addition the epoxide can be conjugated by glutathione transferases (GSTs), which are further metabolized to form aflatoxin-mercapturic acid detoxication products that can be excreted in urine. These products of aflatoxin DNA damage and toxicity as well as other metabolites can be used as biomarkers to evaluate the modulation of aflatoxin activation and detoxication. Levels of the lysine adduct found in serum and the aflatoxin-N7-guanine adduct excreted in urine have been shown to correlate with aflatoxin exposure and to predict liver cancer risk in populations[12]. In animals, changes in hepatic aflatoxin-N7-guanine adduct burdens are reflected in the urinary excretion levels of the adducts, and can be profoundly modulated by chemopreventive interventions [13,14]. As a consequence, these biomarkers have been critical tools for estimating risk of PLC in exposed populations and in the evaluation of chemopreventive agents in both animal models and clinical interventions.

Aflatoxin M1, aflatoxin-N7-guanine and the aflatoxin-albumin adduct have all been used as biomarkers of aflatoxin exposure in studies probing the contribution of aflatoxins to PLC. In a study by Sun and colleagues [6], 145 men with chronic hepatitis B virus residing in Qidong were followed for 13 years. Repeated urine samples were collected prior to

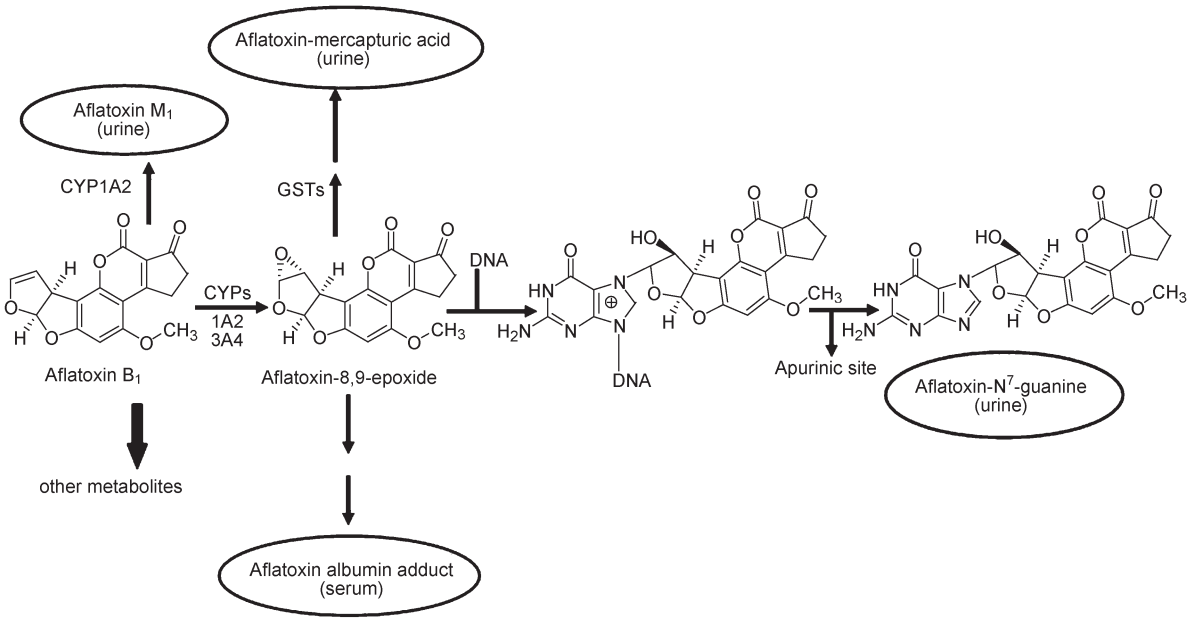

Fig. 1 Aflatoxin B1 metabolism. Aflatoxin-N7-guanine, aflatoxin-mercapturic acid, aflatoxin-albumin adducts, and aflatoxin M1 can be used as biomarkers to evaluate modulation of aflatoxin activation and detoxication in intervention studies and to monitor changes in levels of environmental exposures across time.

initiation of follow-up. The relative risk of PLC was increased 3.5-fold in those with aflatoxin exposure. Interestingly, a similar relative risk was observed using the ser249 TP53 mutation as a potential signature marker of aflatoxin exposure. Many studies conducted outside of Qidong have validated the aflatoxin metabolites as biomarkers of internal dose and risk of PLC (reviewed in [15]).

Cancer prevention trials that use biomarkers as intermediary endpoints provide the ability to assess the efficacy of promising chemopreventive agents in an efficient manner by reducing sample size requirements as well as the time required to conduct the studies compared to trials that have cancer incidence or mortality as endpoints [16]. Because the aflatoxin biomarkers can reflect both the pharmacodynamic action of intervention agents as well as possible risk reduction, they have great utility in chemoprevention trials in aflatoxin endemic areas such as Qidong.

3 Chemoprevention Trials

3.1 Oltipraz

Oltipraz is a drug originally developed for the chemotherapy of schistosomiasis. It is also an effective inhibitor of aflatoxin-induced hepatocarcinogenesis in rats [13]. Pre-clinical and clinical trials have shown that oltipraz modulates the activities of both conjugating/detoxication enzymes as well as cytochrome P450s. For example, a single 125 mg oral dose of oltipraz reduced CYP1A2 activity by 75% in healthy individuals, a cytochrome P450 involved in the bioactivation of aflatoxin B1 [17]. Similar doses also increased GST activity in peripheral lymphocytes, an action that could enhance the detoxication of aflatoxin if also occurring in the liver [18]. A dose-finding study using 125, 250, 500, or 1000 mg/m^2 oltipraz showed increased GST activity in peripheral mononuclear cells and colon mucosa biopsies only at the lower doses [19]. NQO1 RNA transcripts were also increased at 250 mg/m^2. Together, these studies confirm that oltipraz increases cytoprotective enzymes in humans. Phase Ⅱa intervention trials evaluated the modulation of carcinogen metabolism following treatment with oltipraz [20,21]. Participants for a randomized, placebo-controlled, double blind study were recruited from Qidong. Two hundred forty adults with good general health and detectable serum aflatoxin-albumin adduct

levels were randomized to receive placebo, 125 mg oltipraz administered daily or 500 mg oltipraz administered weekly. Urine samples were collected at 2 week intervals during the 8 week intervention period and during an 8 week follow-up period. Urine samples collected after the first month of intervention were assayed for aflatoxin metabolites. These samples were evaluated for alterations in a metabolite reflecting activation, aflatoxin M1, and a detoxication product, aflatoxin-mercapturic acid (Fig. 2). After 1 month of weekly doses of 500 mg oltipraz, the level of aflatoxin M1 excreted in the urine was decreased by 51%. However, the aflatoxin-mercapturic acid levels were not significantly altered. Potential modulation of detoxication enzymes may be masked by inhibition of the activation of aflatoxin B1. The daily administration of 125 mg oltipraz increased aflatoxin-mercapturic acid excretion 2.6-fold, but with only a modest effect on aflatoxin M1 excretion. This trial shows that

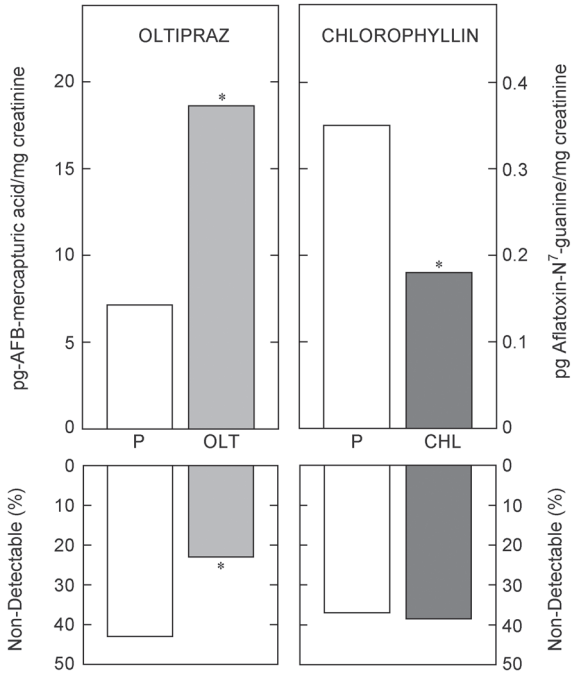

Fig. 2 Mean levels of urinary aflatoxin biomarkers in participants of randomized, placebo-controlled trials with oltipraz (left) and chlorophyllin (right). In these two independent clinical trials, participants received either placebo (P) or 125 mg oltipraz (OLT) once-daily or placebo or 100 mg chlorophyllin (CHL) three times-daily. (*) $P < 0.01$ compared to placebo. Biomarker measures were made on overnight urine samples collected at 4 weeks in the oltipraz trial and 12 weeks in the chlorophyllin trial [21, 26].

induction of cytoprotective genes can be translated into the modulation of aflatoxin disposition in humans. However, cost and availability of the drug, and minor, but dose limiting toxicities of sun sensitivity and pain in the fingertips, render its broad use impractical. Further studies focused on agents found in foods, making them potentially much more accessible and acceptable to the general population. Broad application of chemoprevention in many areas of the world will require approaches of frugal medicine, or what we have termed "green" chemoprevention [22].

3.2 Chlorophyllin

Chlorophylls and their water soluble salts (chlorophyllins) are constituents of the human diet and have been found to be effective anticarcinogens in several animal models [23]. Chlorophyllin is a mixture of sodium-copper salts of chlorophyll that is marketed as an over-the-counter drug. It is also used extensively as a food additive for coloration. Mechanistic studies suggest that chlorophyllin can act as an "interceptor molecule" through the formation of tight molecular complexes with carcinogens such as aflatoxin B1 [24]. Thus, chlorophyllin can diminish the bioavailability of dietary carcinogens by impeding their absorption and by shuttling them through the fecal stream, leading to reduced DNA adduct formation and consequent tumor induction. Chlorophyllin is also a weak inducer of the enzymes involved in the detoxication of aflatoxin [25].

The simple mechanism of molecular complexation, its widespread, low-cost availability, and its lack of any known toxicities prompted us to conduct a randomized, double-blind, placebo-controlled trial in residents of Qidong. One hundred and eighty healthy adults were randomly assigned to ingest 100 mg chlorophyllin or a placebo three times a day for 4 months [26]. The primary endpoint was modulation of aflatoxin-N7-guanine in urine samples collected 3 months into the intervention. Adherence to the study protocol was excellent and no adverse events were reported. As shown in Fig. 2, chlorophyllin consumption at each meal led to an overall 55% reduction in median levels of this aflatoxin biomarker compared to levels in those taking the placebo. In a small, efficient follow-up study Jubert

et al. [27] examined the effects of chlorophyllin and chlorophyll on microdoses of radiolabeled aflatoxin. As seen in the larger clinical trial, substantive reduction in the bioavailability of aflatoxin was observed. Thus, interventions with chlorophyllin or supplementation of diets with foods rich in chlorophylls may represent practical means to prevent the development of PLC and other environmentally-induced cancers.

3.3 Broccoli sprouts

Broccoli sprouts contain an abundance of glucosinolates and isothiocyanates, making them an attractive food-based candidate for chemoprevention. Clinical studies have evaluated metabolism, safety, tolerance, and biomarkers of carcinogenesis using broccoli sprouts [28,29]. Evaluation of broccoli sprout preparations has shown that isothiocyanates are approximately 6 times more bioavailable than the precursor glucosinolates. A placebo-controlled, double-blind, randomized phase Ⅰ clinical study evaluated broccoli sprout preparations containing either glucosinolates or isothiocyanates (principally sulforaphane). The treatment groups received doses of 25 μmol glucosinolates, 100 μmol glucosinolates, or 25 μmol isothiocyanate [30]. No significant or consistent toxicities were observed with any of the broccoli sprout preparations. Interventions using hot water infusions of broccoli sprouts were evaluated in residents of Qidong [29,31]. Modulation of the disposition of aflatoxin was evaluated. Two hundred healthy adults drank infusions of either 400 μmol glucoraphanin or a placebo beverage nightly for 2 weeks. Again, no problems with safety or tolerance were observed. Urinary aflatoxin-DNA adducts were not significantly different between the two interventions. A modest 9% decrease was observed. However, measurement of urinary sulforaphane metabolites showed striking interindividual differences in bioavailability. Between 2% and 50% of the administered glucoraphanin was hydrolyzed to yield sulforaphane, absorbed and then excreted as metabolites. Further analysis to control for the bioavailability of sulforaphane within individuals showed a highly significant inverse association between the levels of sulforaphane metabolites excreted and aflatoxin-DNA adducts (Fig. 3). Thus, if the chemopreventive agent was bioavailable, those individuals tended to have lower levels of the aflatoxin-DNA adduct biomarker. The reduction of aflatoxin-DNA adducts is most likely due to induction of GST activity by sulforaphane. This study shows that aflatoxin disposition can be altered by the administration of glucosinolate-rich broccoli sprout preparations. Future studies with broccoli sprouts will require preparations which produce a higher yield and consistent level of sulforaphane bioavailability.

4 Primary prevention

Maize was the major food vector for aflatoxin exposure. Unlike the rest of Jiangsu province, no rice is grown in Qidong or Haimen counties because of unsuitable soil conditions [32]. Maize consumption in the 1970s, along with occupation of "peasant" and chronic infection with HBV had been noted as major risk factors for liver cancer in the neighboring Haimen county in a study conducted in the 1990s [33]. Interestingly, surveys conducted in the 1970s indicated high percentages of ducks bearing liver tumors, as assessed by pathologists at the Qidong Liver Cancer Institute [2]. Follow-up studies in which ducks and rats were fed maize procured in Qidong, but housed in a distant city, exhibited very high incidences of liver cancer: 12/50 and 24/36 after 2 years for ducks

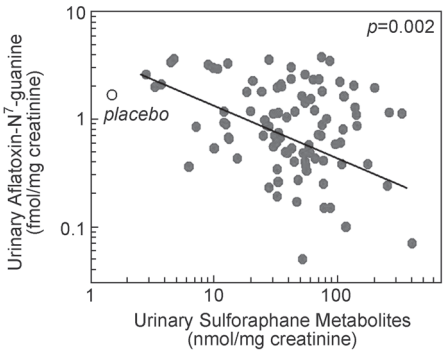

Fig. 3 Scatterplot of urinary levels of aflatoxin-N7-guanine versus sulforaphane metabolites on day 10 of an intervention with glucoraphanin-rich broccoli sprout beverage. (○) Mean level of 99 participants in placebo arm; (●) individual levels from participants randomized to receive the broccoli sprout beverage nightly [31].

and rats, respectively [2]. Annual surveys of aflatoxin contamination in maize in Qidong between 1973 and 1982 indicated that 26% to 99% of acquired samples tested positive for aflatoxin at levels > 20 ppb [2], which is the US Food and Drug Administration action level. The high levels of contamination reflect the extended heat and humidity of this coastal region, both of which promote the growth of the mold *Aspergillus flavus*. This mold in turn produces aflatoxin, both in crops in the field and during post-harvest storage in bins under platform beds in the farm homes [2]. Average annual per capita maize consumption ranged from 82—124 kg during 1973—1982; most Qidong families at that time used maize as their primary dietary staple. At this point a sharp transition occurred because of a new open policy of provisionment in China. By 1998, only 9% of families in the Qidong area ate any maize; <1% ate 100 kg/year. Very little maize is consumed in the Qidong region in 2012. Conversely, the proportion of the rural residents of Qidong consuming some rice reached 97.4% in 1986 and 99.2% in 1997 [34]. After 1979, China adopted institutional and economic reforms to shift from a planned economy to a market-oriented economy. Importation from surrounding areas of new foodstuffs, notably rice, was facilitated into the Qidong region beginning in 1985. Rice is grown in abundance south of the Yangtze River [32].

Archives of serum samples from these clinical trials as well as other studies have provided the opportunity to retrospectively reconstruct levels of aflatoxin exposures in Qidong over the past quarter century. The aflatoxin-albumin adduct is particularly suited for such exposure assessments because of its relatively long half-life (~3 weeks) which allows for an integrated perspective on exposure levels. Moreover, previous studies have shown the aflatoxin-albumin adduct to be stable in frozen serum samples for at least two decades[35]. By contrast, the aflatoxin-N7-guanine adduct has a biological half-life of 6 −8 hours, and only reflects very recent exposures. Randomly selected subsets of these archived serum samples have been analyzed using a very sensitive and specific isotope-dilution mass spectrometry method recently developed by our group [35]. While measures of aflatoxin biomarkers had been performed in many of these cohort samples previously, they monitored different metabolites using a generational spectrum of analytical methodologies. Using this state-of-the-art mass spectrometry approach across the timeline of all collections, it is clear that there has been a stunning >40-fold drop in levels of exposures from the 1980s to the present. As shown in Fig. 4, median levels of aflatoxin-albumin adducts from residents of the villages of Daxin and HeZuo declined from 19.3 pg/mg albumin in 1989, to 3.6 in 1995, to 2.3 in 1999, to 1.4 in 2003 and undetectable in 2009 and 2012. Only 23% and 8% of serum samples had detectable levels (> 0.5 pg/mg allbumin) of this internal dose biomarker in 2009 and 2012, respectively [36]. From these data, it is estimated that the population attributable risk of aflatoxin-related PLC has declined from 47% in 1995 to 11% in 2012.

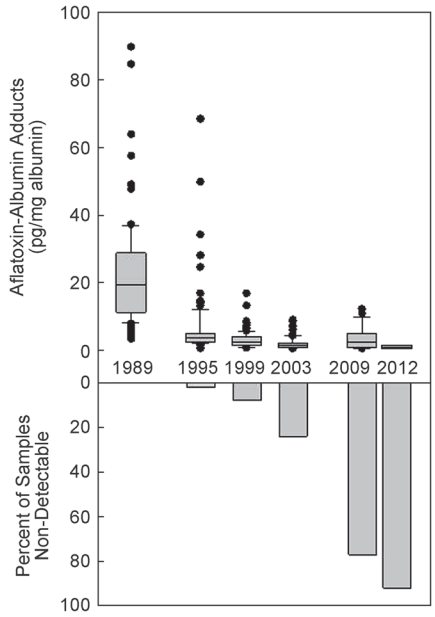

Fig. 4 Box plots of the distributions of aflatoxin-albumin adducts in serum samples collected from healthy rural residents of Qidong from the year 1989 to 2012. Between 75 and 100 samples were analyzed for levels of this internal dose biomarker in each year depicted [36].

5 Conclusions

Why is this important? The biomarker studies provide proof-of-principle that strategies to reduce aflatoxin levels, whether as external exposures in foods or internal dose in humans, can dramatically reduce risk

for developing PLC. There are multiple approaches to attenuating exposures to aflatoxins, including planting pest-resistant varieties of staple crops, reducing mold growth in harvested crops and improving storage methods following harvest. Chemopreventive interventions have also been shown to favorably modulate the metabolism and elimination of aflatoxins in the context of the clinical trials described above. The importance lies in the observations that reductions in aflatoxin exposure leads to rapid declines in mortality rates from PLC in Qidong——within 1 to 2 decades [37]. This rapid efficacy is in contrast to the timeline for impact of HBV vaccination, where it will take more than a generation to substantively reduce the at-risk population (i.e., those over age 35) of this risk factor, given the typical perinatal acquisition of initial HBV infection. Universal, subsidized HBV vaccination of newborns has only been in effect in Qidong for the past decade. Elimination of all risk factors through vaccination, dissipation of maize consumption, economic development and concomitant better and diverse food options, and perhaps chemoprevention, will render liver cancer to the ranks of a rare disease in Qidong in the coming decades. Unfortunately, new cancers are emerging to take its place at the top of the cancer mortality tables. Lung, breast and colon cancer rates are rising with the emerging lifestyle, dietary and economic changes. Similar paradigms of using biomarkers to understand etiologies and evaluate the efficacy of primary and chemopreventive interventions will again be on center stage for these emergent challenges.

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中国启东肝癌预防：黄曲霉毒素生物标志物研究的经验

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摘 要 原发性肝癌一直是中国启东癌症死亡的首要原因，这一地区的110万居民中大约每年有800人死于原发性肝癌。流行病学研究已经强调了乙肝病毒感染和肝致癌物黄曲霉素的饮食暴露在启东和其他流行地区作为关键相互作用的风险决定因素的重要性。由于生物标志物的发展，使得在病例对照研究和其他群体研究中可以检测这些风险因素的流行程度。20世纪80年代曾经在启东进行了乙肝病毒疫苗接种试点，但直到本世纪初才在新生儿上普遍接种。尽管目前疫苗接种对癌症死亡率的影响微乎其微，但该项目旨在减缓这一地区未来几代人肝脏疾病包括肝癌的发展。减少对黄曲霉毒素暴露的策略也是必需的，尤其是对于那些已经感染乙肝病毒的人群。我们已经进行了一系列原理验证性的临床试验，包括使用药物、膳食补充剂和食物来改变不可避免的暴露后黄曲霉素的代谢及排出。使用黄曲霉素生物标志物作为中间指标，奥替普拉、叶绿素和西兰花豆芽饮料已被证明有效，突出了低成本化学预防途径在预防环境致癌中的作用。值得注意的是，最近对存档的血清样本的回顾性分析表明，启东的黄曲霉素暴露在过去的25年里下降了40倍，这可能是由从玉米到大米的主食变化带来的。因此，一级预防，伴随着20世纪80年代经济和农业政策改变的作用，带来了出乎意料的在短时间内消除这个地区肝癌地方性流行的希望。

关键词 黄曲霉毒素-N7鸟嘌呤 黄曲霉素-白蛋白加合物 原发肝癌 化学预防

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