Accepted Manuscript

Title: The association between exposure to aflatoxin, mutation in *TP53*, infection with hepatitis B virus, and occurrence of liver disease in a selected population in Hyderabad, India

Author: S. Anitha D. Raghunadharao F. Waliyar H. Sudini M. Parveen Ratna Rao P. Lava Kumar

PII: S1383-5718(14)00067-9
DOI: http://dx.doi.org/doi:10.1016/j.mrgentox.2013.12.011
Reference: MUTGEN 402453

To appear in: *Mutation Research*

Received date: 26-7-2013
Revised date: 30-11-2013
Accepted date: 28-12-2013

Please cite this article as: S. Anitha, D. Raghunadharao, F. Waliyar, H. Sudini, M. Parveen, R. Rao, P.L. Kumar, The association between exposure to aflatoxin, mutation in *TP53*, infection with hepatitis B virus, and occurrence of liver disease in a selected population in Hyderabad, India, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* (2014), http://dx.doi.org/10.1016/j.mrgentox.2013.12.011

The association between exposure to aflatoxin, mutation in *TP53*, infection with hepatitis B virus, and occurrence of liver disease in a selected population in Hyderabad, India

S. Anitha[a,†,*], D. Raghunadharao[b], F. Waliyar[c], H. Sudini[a], M. Parveen[d], Ratna Rao[e], P. Lava Kumar[f]

[a] *International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Andhra Pradesh, India*

[b] *Department of Medical Oncology, Nizam's Institute of Medical Sciences (NIMS), Hyderabad 500 082, India*

[c] *ICRISAT, Niamey BP 12404, Niger*

[d] *Department of Medical Gastroenterology, PGIMS, Rohtak, 124001, India*

[e] *Department of Microbiology and Immunoserology, Apollo Health City, Jubilee Hills, Hyderabad 500 033, India*

[f] *International Institute of Tropical agriculture (IITA), PMB 5320, Ibadan, Nigeria*

[†] *Present address: ICRISAT, PO BOX 1096, Lilongwe, Malawi*

***Corresponding author:**

S. Anitha, PhD

Pathology and Molecular Diagnostics Laboratory

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT)

PO BOX 1096, Lilongwe, Malawi

Tel. No. (+265) 0994081714

E-mail: s.anitha@cgiar.org

Abstract

Aflatoxin B1 is a carcinogen produced by *Aspergillus flavus* and a few related fungi that are often present in many food substances. It interacts synergistically with Hepatitis B or C virus (HBV, HBC) infection, thereby increasing the risk of hepatocellular carcinoma (HCC). The G to T transversion at the third position of codon 249 (AGG) of the *TP53* gene, substituting arginine to serine, is the most common aflatoxin-induced mutation linked to HCC. This study examined

mutations in *TP53* by PCR-RFLP analysis and by measurement of an aflatoxin-albumin adduct as a biomarker for human exposure of aflatoxin B1 by indirect-competitive ELISA, in samples collected from healthy controls as well as patients with hepatitis in Hyderabad, Andhra Pradesh, India. A total of 238 blood samples were analyzed the presence of the G to T mutation. Eighteen of these samples were from HBV-positive subjects, 112 of these were from subjects who had HBV-induced liver cirrhosis, and 108 samples were taken from subjects without HBV infection or liver cirrhosis (control group). The G to T mutation was detected in 10 samples, 8 of which were from subjects positive to both HBV and aflatoxin-albumin adduct in blood ($p = 0.07$); whilst two were from individuals who were HBV-negative, but positive for the aflatoxin-albumin adduct ($p = 0.14$). The aflatoxin-albumin adduct was detected in 37 of 238 samples, 29 samples were from HBV-positive subjects and eight were from individuals who were positive for both HBV and the *TP53* mutation ($p=0.07$). The concentration of aflatoxin-albumin adduct ranged from 2.5 to 667 pg/mg albumin. Despite low incidence of the G to T mutation, its detection in subjects positive to aflatoxin-adducts is indicative of a strong association between the mutation and aflatoxin exposure in India.

Keywords:
Biomarker, *TP53* mutation, aflatoxins, hepatitis virus, hepatocellular carcinoma

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world, accounting for about 5% of all human cancers, and the second cause of death from cancer worldwide [1]. It is the fifth most common cancer in men and the eighth most common cancer in women [2]. The incidence of HCC in India is reported to be between 0.2 and 1.9% [3]. Infection with hepatitis B or C virus is considered to be the major cause for HCC. The hepatitis B virus (HBV) is highly prevalent in the developing world, with an estimated two billion people infected. In India, HBV prevalence among the general population ranges from 2% to 8%, placing India in the intermediate HBV-endemic zone, where the number of HBV carriers is estimated to be 50 million, forming the second largest global pool of chronic HBV infections [4]. Previous studies have implicated a multifaceted etiology for human HCC [5]. In addition to chronic infection with hepatitis B or C viruses and lifestyle habits (such as alcohol abuse), dietary exposure to aflatoxin B1 (AFB1) is a major risk factor for development of human HCC [5-6], especially where exposure to HBV is endemic.

Mycotoxins are toxins produced by certain fungi, and aflatoxins are an important group of mycotoxins produced by the fungi *Aspergillus flavus* and *A. parasiticus*, which contaminate staple foods including groundnuts [2]. The etiologic involvement of AFB1 in human HCC can be inferred from the high incidence of this disease in certain geographical regions, particularly, South and Southeast Asia and sub-Saharan Africa, where the warm and humid climate promotes mold growth and contamination of food supplies by this naturally occurring mycotoxin [5-6]. Several studies have shown that aflatoxin contamination is frequent in food crops grown in Asia and sub-Saharan Africa [7]. AFB1 is metabolized by P450 enzymes in the liver and generates an epoxide that is highly reactive with DNA, forming adducts at the N7 position of guanine. If not repaired, these lesions may lead to permanent DNA mutations, preferentially G to T transversions [2]. The epoxides are detoxified to a limited extent by glutathione *S*-transferase (GST)-mediated conjugation with reduced glutathione (GSH) to form AFB1 *exo*- and *endo*-epoxide–GSH conjugates [8]. The most common aflatoxin-induced mutational hot spot found in HCCs in more than 50% of cases reported from different geographical regions, such as China and southern Africa, is a G to T transversion at the third position of codon 249 (AGG) of the *TP53* gene, which results in the substitution of arginine by serine [1,9]. However, very little is known about the prevalence of aflatoxin exposure and its impact on liver disorders in HBV-

positive patients in India. Earlier reports on liver-biopsy samples suggest that the frequency of *TP53* mutation is low in India [10]. Another study measured AFB1 exposure in 4.5% of HBV-positive samples in India [11].

This study was undertaken to determine the association between infection with HBV, exposure to aflatoxin, and mutation in codon 249 of *TP53*, in blood samples of patients with different stages of liver disease. This work is based on a model for end-stage liver disease (MELD) in 130 subjects positive for HBV and/or suffering from liver cirrhosis, and 108 samples from a healthy control group (hepatitis B- or C-negative, no liver disorder).

2. Materials and methods

2.1. Sample collection

Samples were collected from two hospitals in Hyderabad during the years 2009 and 2010. Patients with clinically confirmed liver disorders (decompensated liver disease) were selected for this study. A total of 238 blood samples were collected, of which 130 blood samples were from patients with liver diseases, and 108 samples were from the control group comprising individuals living in the same household as the diseased subject, but without any history of liver disease. Demographic data (age, sex, address, clinical signs), family history of liver disease, lifestyle habits such as tobacco use and alcohol consumption, dietary profile, clinical and laboratory profiles including results from liver-function tests, were recorded. Clinical and dietary profiles of each patient were documented by use of a standard questionnaire. The clinical profile of a subject was used to identify the stage of liver disease with the 'Model for end-stage liver disease' (MELD) and 'Child Turcotte Pugh' (CTP) scoring systems [12]. All the objectives of the study were clearly explained to the participating individuals, and written consent was obtained before collecting blood samples. The study was approved by the Ethics Committee of the Nizam's Institute of Medical Sciences, Hyderabad.

2.2. Scoring the severity of liver disease based on clinical profile

MELD and CTP scoring-systems were used to determine the severity of liver disease. Based on liver-function tests for serum creatinine, total bilirubin, and international normalized ratio (INR), a MELD score was calculated to quantify the end-stage liver disease for each patient with the following scoring equation proposed by The Mayo Clinic group [13]:

3.8Lg[total bilirubin (mg/dl)] + 11.12 Lg(INR) + 9.6 Lg[creatinine (mg/dl)] + 6.4 (Aetiology: 0 if cholestatic or alcoholic, 1 if otherwise).

The CTP scoring-system involves allotting points for albumin level, ascites, total bilirubin, prothrombin time and hepatic encephalopathy, and scores can vary from 5–15 (Table 1). Based on total score, patients are designated as Child's class A (CTP 5–6), class B (CTP 7–10) and class C (CTP 11–15).

2.3. Detection of TP53 *mutation by PCR amplification and restriction-enzyme digestion*

DNA was extracted from blood samples that were taken from the subjects and collected in EDTA vials. Samples were kept in an ice-box and transferred to the Mycotoxicology and Virology lab at ICRISAT, Hyderabad, where they were stored at -20ºC until processing time. DNA was extracted from 200 µl of blood by use of a commercial DNA-extraction kit (Nucleospin Blood, Cat # 740951, Macherey Nagel, Germany) according to the manufacturer's protocol. DNA was used for the amplification of a 254-bp portion of exon 7 surrounding codon 249 by means of the polymerase chain-reaction (PCR) with primer pair p53Fser (5'CTT GCC ACA GGT CTC CCC AA 3') and p53Rser (5'AGG GGT CAG RGG CAA GCA GA 3'), similar to the primers used in earlier work in China [14]. PCR was performed in a thermal cycler (Applied Biosystems) with an initial denaturation step of 2 min at 94°C followed by 40 cycles of denaturation at 94°C for 30 seconds (s), annealing at 60°C for 30s, and extension at 72°C for 30s. The amplified product (6 µl) was digested with restriction enzyme *Hae* III (New England Biolabs, UK) at 37˚C for 4 h. PCR products and *Hae* III-digested products were analyzed by means of electrophoresis in a 2% TAE-agarose gel. The gels were stained with ethidium bromide and visualized on a UV-transilluminator.

2.4. Measurement of aflatoxin-albumin adducts

The AFB1-lysine (AFB1–lys) adduct found in human serum-albumin (HSA) was used as a biomarker to assess exposure to aflatoxin of the subjects [11]. Five ml of blood sample were collected in plain vacutainers from 238 individuals. Serum was separated from each sample and processed to measure the amount of AFB1–lys adduct by an indirect competitive enzyme-linked immunosorbent assay (IC-ELISA) essentially as described by Anitha *et al*. [11]. This procedure involves extraction of the albumin fraction from 500 µl of serum, and measurement of its concentration according to Bradford [15]. Two mg of albumin was digested with proteinase K, and the digested product was analyzed in an IC-ELISA with an antibody produced against AFB1–BSA conjugate [11]. Alkaline phosphatase-conjugated anti-rabbit antibodies (AR-ALP) were used as a secondary antibody, and *p*-nitrophenyl phosphate (pNPP) was applied as a substrate. The colorimetric reaction was followed on an ELISA plate-reader with a 405-nm filter.

2.5. Statistical Analysis

Samples were collected from 238 individuals with clinical profiles, food (diet) consumption-related questionnaires and demographic data. These data were entered in a database (SAS 9.2V) and used for statistical analysis with SAS 9.2V. Pearson's chi-square and Fisher's exact tests were applied to assess the statistical significance of the frequency of independent variables and codon-249Ser mutations, by means of the software SAS 9.2V. This helped to estimate the risk of mutation among the study groups considering age, sex, HBV surface-antigen status, and aflatoxin-exposure status as potential confounders.

3. Results

A total of 238 blood samples were analyzed for the presence of *TP53* gene mutations. The age of the subjects (64% men) ranged from 36 to 61 years, 130 individuals were HBV-positive and 108 negative. Among the HBV-positive samples, 112 were from subjects with liver cirrhosis. The use of PCR primers p53Fser and p53Rser resulted in amplification of a 254-bp product in all 238 samples (Fig. 1). The presence of a mutation in codon 249 was assessed by RFLP of the PCR product, by use of the *Hae* III enzyme that cleaves the GG$^{\downarrow}$CC sequence at codon position 249-250, generating products of 92 bp, 66 bp and several smaller fragments from

the 254-bp PCR product. In samples with a G→T mutation at codon 249, *Hae* III digestion yields a 158-bp fragment due to loss of a restriction site (Fig. 1).

The G→T mutation at codon 249 of the *TP53* gene was detected in 10 samples (4.2%), eight of which were from subjects infected with HBV and suffering from liver cirrhosis, and who also had aflatoxin-albumin adduct (p = 0.07); the other two were from control subjects (HBV-negative, no liver disease) who were positive for the AFB1–1ys adduct (p = 0.14). In 37 of 238 samples analyzed, the AFB1–lys adduct was found in the IC-ELISA. Among these, 29 samples were from subjects who had cirrhosis of the liver and were positive for HBV, and eight samples were from individuals who were positive for both HBV and mutation in *TP53* (p=0.07).

Of ten samples positive for mutation at codon 249 of *TP53*, two were from the control group, five samples were in the Child-B and three in the Child-C category, with the highest MELD scores with decompensated liver disease (Table 2). These ten samples also had a high amount of AFB1–lys adducts (>76 pg/mg albumin) (Table 3). With a set p-value of 0.1, aflatoxin exposure (AFB1–lys) and *TP53* mutation were dependent on each other, otherwise they were independent (Table 4). This shows that *TP53* mutation and AFB1–lys adduct are dependent in HBV samples. Among the 37 individuals who provided AFB1–lys-positive samples, eight were from the control group; 18 individuals were infected with HBV but did not show any clinical condition and fell in the Child-A category with initial stages of cirrhosis based on CTP and MELD scores; eight individuals were positive for HBV and cirrhosis of the liver and fell in the Child-B category with decompensated liver disease based on CTP and MELD scores; two had cirrhosis without HBV infection and fell in the Child-C category with decompensated liver disease; and one was infected with HBV, suffered from cirrhosis and fell in the Child-C category with decompensated liver disease (Table 2).

There was no significant difference in the 249Ser mutation level between females with HBV (p=0.12) and males with HBV (p=0.38). This shows that sex has no influence on the presence of aflatoxin and *TP53* mutation. Similarly, daily consumption of rice and the presence of AFB1–lys with *TP53* mutation in HBV-positive individuals had no significant influence (p=0.19). With a set p-value of 0.1, the influence of aflatoxin exposure and *TP53* mutation in non-alcoholic individuals with liver disease and HBV-positive status were dependent on each other, otherwise they were independent (p=0.05) (Table 4). The concentration of the AFB1–lys adduct ranged from 2.5 to 677 pg/mg albumin with a mean adduct level of 52.1 pg/mg.

Interestingly, the individuals positive for both HBV and *TP53* mutation had an AFB1–lys level > 75 pg/mg albumin (Table 3). This level was higher than that of individuals positive for HBV and without mutation (< 75 pg/mg albumin). The MELD score showed that the presence of HBV together with AFB1–lys produced a higher mutation percentage, and all patients had decompensated liver disease (Table 2).

4. Discussion

Carcinogenesis is a multistep process, and the interaction of host-cell factors along with viral and chemical carcinogens appears to play a key role in the development of hepatocellular carcinoma in humans [10]. When the liver is exposed to HBV, the hepatocytes are injured and undergo structural changes [15]. Dietary exposure to AFB1 leads to the formation of aflatoxin epoxide, which is highly reactive and binds to albumin, which is a major plasma protein produced in liver cells. It also binds to DNA, forming adducts at the N7 position of guanine [16-17]. A lack of repair may give rise to mutations in the *TP53* gene, which may lead to the loss of its tumour-suppressor function and uncontrolled proliferation of liver cells, which may result in HCC. The transformation of a normal cell to a cancercell is a complex process, accompanied by multiple steps of genetic and epigenetic alterations [18]. Although a diverse set of genes has been implicated in tumorigenesis, the transcription factor p53 (encoded by the human gene *TP53*) stands out as a key tumour-suppressor gene [19]. The role of *TP53* as a tumour-suppressor gene includes the ability to induce cell cycle arrest, DNA repair, senescence, and apoptosis [20]. The *TP53* gene in human tumours is often found to undergo missense mutation, where a single nucleotide is substituted with another [21]. Consequently, a full-length protein containing only a single amino-acid substitution is produced, which results in loss of its suppressor activity. It is well established that p53 inactivation and expression of mutant p53 can confer cells with additive growth and survival advantages, such as increased proliferation, evasion of apoptosis, and chemo-resistance [18].

Globally, 600,000 people die every year due to infection with Hepatitis B [22]. In the Middle-East and on the Indian sub-continent, 2–5% of the general population is chronically infected with Hepatitis B virus [22]. In India, few studies previously indicated that AFB1 exposure is one of the important and possible risks for liver cancers [23]. However no study has been conducted in India to map the associations among aflatoxin exposure, *TP53* mutations and

liver diseases in tandem. In our study, we made an attempt to look into these complex relationships. Surprisingly, of 238 patients analyzed, 10 patients were positive for *TP53* mutation, and all of them were positive for AFB1–lys, an indication of aflatoxin exposure. Frequencies of aflatoxin exposure, *TP53* mutation and HBV, with p-values in the Chi-square test and in Fisher's exact test were analysed (Table 4). There were eight samples from individuals with high aflatoxin exposure (protein adduct level >76 pg/mg albumin) and *TP53* mutation and two samples from subjects with low aflatoxin-protein levels (<76 pg/mg albumin) (Table 3). This shows that the level of AFB1–lys may raise the probability of obtaining a *TP53* mutation. Although the number of mutations is low, it is still considerable when taking 138 cirrhosis samples into consideration. In the study group, 216 individuals were non-alcoholic. Among them, 23 HBV-positive individuals had the AFB1–lys adduct in their blood and eight of them had a *TP53* mutation (p=0.05) (Table 4). Among the 22 alcoholics, not one had a *TP53* mutation. These data indicate a link between the occurrence of a mutation at codon 249 with the presence of aflatoxin-albumin adduct in blood, which is an indication of exposure to dietary aflatoxins.

No significant correlation was observed between consumption of rice and the presence of aflatoxin-albumin adducts with *TP53* mutation (Table 4). People in southern India consume diverse foods apart from rice (corn, wheat, chillies and groundnuts) every day. This may have a cumulative effect on the presence of aflatoxin-albumin adduct in the blood. It is difficult to determine the exact contributing dietary source of aflatoxin unless the foods themselves are analyzed for aflatoxin.

The results of the present study show an association between HBV and AFB1 in patients with liver disorders. An earlier study conducted in India [10] on *TP53* tumour-suppressor gene mutations in 21 patients with HCC, revealed a low frequency (9.5%; 95% CI, 22.1 – 23.0) of *TP53* gene mutation at codon 249, the most common mutational hot spot in HCC. A low frequency of *TP53* mutation involving codon 249 has also been reported in Germany, Taiwan, and Thailand [24-26]. The levels of dietary exposure to aflatoxin in Thailand and the presence of the mutated DNA in plasma is consistent with the notion that exposure to this toxin, together with chronic HBV infection remains a major etiological factor for HCC in Thailand [2]. The aflatoxin level in India was high in non-carriers of HBV, which is indicative of the independent role played by AFB1 in hepatocarcinogenesis [3]. With 68.7% of the non-B non-C hepatitis

patients having tissue localization of AFB1, and the concomitant presence of AFB1 along with HBV in 46.1% of cases studied, HBV is an additional risk factor to which populations from developing countries are exposed, and it plays an aggravating role in hepatocarcinogenesis. This statement is supported by the current study in which 22% of HBV-positive cases had aflatoxin-albumin adduct, of which 8% had a *TP53* mutation. Interestingly, 7.4 % of individuals in the healthy control group (HBV-negative, no liver disease) had the aflatoxin-albumin adduct and 1.8% of these had a *TP53* mutation, with a high aflatoxin-albumin level, ranging between 300-600 pg/mg albumin.

The MELD and CTP scores were used to identify the severity of liver disease. MELD and CTP scores are complementary to each other in evaluating the progressive clinical condition of liver cirrhosis [27]. They also provide the advantage of ranking patients according to the severity of the underlying liver disease and risk of mortality [12]. The total CTP score ranged between 5 and 15. Those classified as CTP 5-6 fall under Child's class A, CTP 7-10 fall under Child's class B and CTP 11-15 falls under Child's class C (Table 1). The survival of individuals decreases with a Child's score of 7 or greater, i.e., Child's class B or C. The scoring is done based on hepatic encephalopathy, presence of ascites, albumin, total bilirubin level, prothrombin time and international normalized ratio (INR) for prothrombin time.

The MELD score was calculated by use of serum creatinine, total bilirubin, INR and underlying etiology for end-stage liver disease. Status-1 patients are those with the highest MELD score and where the liver is considered severely decompensated. The presence of aflatoxin-albumin, together with hepatitis B and cirrhosis of the liver, increases the severity of liver disease that can be interpreted from different stages and severity (Child A/B/C) based on CTP and MELD score (Table 2).

The MELD and CTP scores were compared with the presence of aflatoxin and *TP53* mutation, along with Hepatitis B infection. The results show that there is critical link between the aflatoxin presence together with Hepatitis B and cirrhosis of the liver to elicit and increase the severity of liver disease (Table 2). Whenever HBV infection or exposure to aflatoxin occur on their own, and if detected at an early stage, there may be the possibility of reducing the progress of Child A to Child C with antiviral treatment. When the liver stage is in the Child-C category, it is an indication for liver transplantation or mortality [12]. The data show that most of the end-stage liver disease and CTP scores are linked with the presence of aflatoxin and HBV, which

indicates that these two together increase the severity of the liver disease. Finally, on the one hand it is very important to have good biomarker system for the detection of aflatoxin exposure and its deleterious effects on human health. On the other hand, it is always important to raise the awareness levels among different stakeholders and to have proper regulatory limits to restrict the free allowance of aflatoxin-contaminated products into the food chain. The proper management of controlling aflatoxin exposure at an early stage would help reduce the chance of getting a *TP53* mutation, and the patient would respond to the treatment to reduce the viral load to reduce the severity of the liver disease.

6. Conclusions

This study compared 238 individuals with (130 HBV-positive) or without liver disease (108 HBV-negative) by measuring the frequency of *TP53* mutation at codon 249 and the level of aflatoxin B1 adducts in albumin. Among the 130 HBV-positive patients, eight were positive for the *TP53* mutation and 29 for aflatoxin-albumin adducts. Serum aflatoxin-albumin adducts, and mutation at position 249 in the *TP53* gene were detected in the DNA of blood samples from patients with HBV infection and liver disorder. The severity of the liver disease with HBV weakly correlated with the presence and the level of aflatoxin-albumin adducts and with *TP53* mutation at codon 249 ($p=0.07$).

Conflict of interests statement

The authors declare no conflict of interests.

Acknowledgements

We acknowledge ICRISAT for financial and logistical support of this study. We also thank the Statistics Department of ICRISAT for help with the data analysis.

References

[1] A. Behnoush, G. Doriane, V. Stephanie, S. Masoud, H. Pierre, TP53 Mutations and HBX status analysis in hepatocellular carcinomas from Iran: evidence for lack of association between HBV genotype D and TP53 R249S mutations, Hep. Res. Trea. 2011 (2011) 1-6.

[2] S. Villar, O. Sandra, A. Behnoush, G. Doriane, N. Andre, P. Amelie, K. Thiravud, K. Anant, S. Suleeporn, D.F. Marlin, J.D. Groopman, P. Hainaut, Aflatoxin-Induced TP53 R249S Mutation in Hepato Cellular Carcinoma in Thailand: Association with Tumors Developing in the Absence of Liver Cirrhosis, PLosone. 7 (2012) 1-7.

[3] K.G. Murugavel, P.P. Naranatt, E.M. Shankar, S. Mathews, K. Raghuram, P. Rajasambandam, V. Jayanthi, R. Surendran, A. Murali, U. Srinivas, K.R. Palaniswamy, D. Srikumari, S. P. Thyagarajan, Prevalence of aflatoxin B1 in liver biopsies of proven hepatocellular carcinoma in India determined by an in-house immunoperoxidase test, J. Med. Microbiol. 56 (2007) 1455-1459.

[4] S. Datta, An overview of molecular epidemiology of hepatitis B virus (HBV) in India, Virology Journal. 5 (2008) 1-12.

[5] M.C. Yu, J.M. Yuan, Environmental factors and risk for hepatocellular carcinoma, Gastroenterol. 127 (2004) S72-S78.

[6] F.X. Bosch, J. Ribes, M. Diaz, R. Cleries, Primary liver cancer: worldwide incidence and trends, Gastroenterology. 127 (2004) S5-S16.

[7] F. Waliyar, D. Traore, D. Fatondji, B.R. Ntare, Effect of irrigation interval, planting date, and cultivar on *Aspergillus flavus* and aflatoxin contamination of peanut in a sandy soil of Niger. Peanut Science 30 (2003) 79-84.

[8] C.P.Wild, P.C.Turner, The toxicology of afaltoxins as a basis for public health decisions, Mutagenesis. 17 (2002) 471-481.

[9] A. Besaratinia, S.I. Kim, P. Hainaut, G.P. Pfeifer, In vitro recapitulating of TP53 mutagenesis in hepatocellular carcinoma associated with dietary aflatoxin B1 exposure, Gastroenterol. 137 (2009) 1127-1137.

[10] S. Katiyar, B. Dash, V. Thakur, R.C. Guptan, S.K. Sarin, B.C. Das, p53 Tumor suppressor gene mutations in Hepatocellular carcinoma patients in India, Cancer. 88 (2000) 1565-1573

[11] S. Anitha, F. Waliyar, A.S. Reddy, Ratna Rao, D. Raghunadharao, P.L. Kumar, Development of a simple enzyme-linked immunosorbent assay for quantitative estimation of aflatoxin B_1 adduct in humans, Curr. Sci. 101 (2011) 844-846.

[12] R. Verma, B. Emmet, Keeffe, A. Ahmed, MELD score: The implementation of evidence-based selection criteria for allocation of donor livers, Pract. Gastroenterol.27 (2003) 13-21.

[13] F. Botta, E. Giannini, P. Romagnoli, A. Fasoli, F. Malfatti, B. Chiarbonello, E. Testa, D. Risso, G. Colla, R. Testa, MELD scoring system is useful for predicting prognosis in patients with liver cirrhosis and is correlated with residual liver function: a European study, Gut 52 (2003) 134-139.

[14] H. Xing-Hua, L. Sun, D. Lu, Y. Sun, L. Ma, X. Zhang, J. Huang, L. Yu, Codon 249 mutation in exon 7 of *p53* gene in plasma DNA: maybe a new early diagnostic marker of hepatocellular carcinoma in Qidong risk area, China, World J. Gasteroenterol. 9 (2003) 692-695.

[15] B. Chapot, C.P. Wild, ELISA for quantification of Aflatoxin-albumin adducts and their application to human exposure assessment, Techn. Diag. Patho. 2 (1991) 135-155.

[16] S.W. Baertschi, K.D. Raney, M.P. Stone, T.M. Harris, Preparation of the 8,9-epoxide of the mycotoxin aflatoxin B1: the ultimate carcinogenic species, J. Am. Chem. Soc. 110 (1988) 7929-7931.

[17] M. Walton, P. Egner, P.F. Scholl, J. Walker, T.J. Kensler, J.D. Groopman, Chem. Res. Toxicol. 14 (2001) 919.

[18] N. Rivlin, R. Brosh, M. Oren, V. Rotter,V, Mutation in the p53 Tumor suppressor gene: important milestones at the various steps of tumorigenesis, Genes Cancer. 2 (2011) 466-474.

[19] A.J. Levine, M. Oren M, The first 30 years of p53: growing ever more complex, Nat. Rev Cancer. 9 (2009) 749-758.

[20] Y. Aylon, M.Oren, New plays in the p53 theater, Curr.Opin.Genet. Dev. 21 (2011) 86-92.

[21] P. Hainaut, M. Hollstein, p53 and human cancer: the first ten thousand mutations, Adv Cancer. Res. 77 (2000) 81-137.

[22] WHO 2013. Hepatitis B. Fact sheet # 204 (updated July 2013). World Health Organization. http://www.who.int/mediacentre/factsheets/fs204/en/#

[23] M. Asim, M.P. Sarma, L. Thayumanavan, P. Kar, Role of aflatoxin B1 as a risk for primary liver cancer in north Indian population, Clinical Biochemistry. 44 (2011) 1235-1240.

[24] S. Kress, U.R. Jahn, P. Bannasch, M. Schwarz, p53mutations in hapatocellular carcinoma from Germany, Cancer Res. 52 (1992) 3220-3223.

[25] S. Hosono, C.S. Lee, M.J. Chou, C.S. Yang, C. Shih, Molecular analysis of the p53 alleles in primary hepatocellular carcinomas and cell lines, Oncogene. 6 (1991) 237-43.

[26] M.C. Hollstein, C.P. Wild, F. Bleicher, S. Chutimatawin, C.C. Harris, P. Sivatanakul, p53 mutation and aflatoxin B1 exposure in hepatocellular carcinoma patients from Thailand, Int. J. Cancer. 53 (1993) 51-5.

[27] B. Angermayr, M. Cejna, F. Karnel, M. Gschwantler, F. Koenig, J. Pidlich, H. Mendel, L. Pichler, M. Wichlas, A. Kreil, M. Schmid, A. Ferlitsch, E. Lipinski, H. Brunner, J. Lammer, P. Ferenci, A. Gangl, M. Peck-Radosavljevic, Child-Pugh versus MELD score in predicting survival in patients undergoing transjugular intrahepatic portosystemic shunt, Gut. 52 (2003) 879-885.

Table 1
Severity of liver disease according to the Child Turcotte Pugh (CTP) scoring system

Parameter*	Points**		
	1	2	3
Hepatic encephalopathy	None	Grade 1–2	Grade 3–4
Ascites (peritoneal cavity fluid) measured by abdominal ultrasound	Absent	Slight	Moderate
Serum albumin (mg/dl)	> 3.5	3.5–2.8	< 2.8
Total bilirubin (mg/dl)	< 2	2–3	> 3
International normalized ratio (INR)	< 1.7	1.7–2.3	> 2.3

*Source = liver-function test records of subjects; **Points assigned;
CTP scores range between 5 to 15; CTP 5–6 = Child's class A; CTP 7–10 = Child's class B; CTP 11–15 = Child's class C [see ref 12]

Table 2
TP53 mutation and AFB1-lys adducts in liver-cirrhosis and HBV serum-positive patients grouped by Child Turcotte Pugh (CTP) and Model for End-Stage Liver Disease (MELD) scores

CTP category	Number of samples	MELD Score	Clinical condition	Hepatitis B virus	Number of samples with mutation at position 249 of the *TP53* gene	Number of samples positive for AFB1-lysine	Mean adduct level (pg/mg albumin)
Not applicable (control group)	108	Not applicable (control group)	None	Negative	2	8	19.25
Child's class A	18	Initial stage of liver disorder	None	Positive	0	18	18.1
Child's class B	87	Decompensated liver disease	Cirrhosis of liver	Positive	5	8	71.25
Child's class C	2	Decompensated liver disease	Cirrhosis of liver	Negative	2	2	77.5
Child's class C	23	Decompensated liver disease	Cirrhosis of liver	Positive	1	1	575 [value correct? Ed.]

Table 3
AFB1-lys in human serum albumin detected by IC-ELISA and *TP53* mutation detected by PCR-RFLP

Range of AFB_1-lys adduct concentration (pg/mg albumin)	Number of samples	Number of samples with mutation at position 249 in the *TP53* gene
0[a]	201	-
2.5–25	17	-
26–50	7	-
51–75	4	2
> 76	9	8

[a]No detectable AFB1-lys (optical density values equivalent to negative control)

Table 4
Demographic data of the study group categorized by HBV infection (serum Ag-positive), sex, alcohol consumption, and staple diet

Parameter	No of samples	Mutation at 249 position in p53 gene	AFB1-lys adduct positive	Positive for mutation and AFB1-lys adduct	*P*** Value
Serum Hepatitis B					
Positive	130 (54.6)	8 (6.1)	29 (22.0)	4 (3.0)	0.07
Negative	108 (45.4)	2 (1.8)	8 (7.4)	1 (0.9)	0.14
Sex					
Male					
Hepatitis B-positive	88 (67.6)	7 (7.9)	24 (27.2)	4 (4.5)	0.38
Hepatitis B-negative	65 (60.1)	2 (3)	7 (10.7)	1 (1.5)	0.21
Female					
Hepatitis B-positive	42 (32.3)	1 (1.17)	5 (5.8)	1 (2.3)	0.12
Hepatitis B-negative	43 (39.8)	0 (0)	1 (1.17)	0 (0)	*
Alcohol consumption					
Alcoholic					
Hepatitis B-positive	21 (16.1)	0 (0)	6 (28.5)	0 (0)	*
Hepatitis B-negative	1 (0.92)	0 (0)	0 (0)	0 (0)	*
Non-alcoholic					
Hepatitis B-positive	109 (83.8)	8 (7.3)	23 (21.1)	4 (3.6)	0.05
Hepatitis B-negative	107 (99.0)	2 (1.9)	8 (7.5)	1 (0.9)	0.14
Staple diet					
Rice					
Hepatitis B-positive	117 (90.0)	2 (1.7)	16 (13.6)	2 (1.7)	0.19
Hepatitis B-negative	100 (92.5)	1 (1)	0 (0)	1 (1)	*
Rice and wheat					
Hepatitis B-positive	13 (10.0)	6 (46.1)	13 (100)	4 (30.7)	*
Hepatitis B-negative	8 (7.4)	1 (12.5)	8 (100)	0 (0)	*

Values in parentheses are percentages; *means row or column sum zero (no statistics is computed);** probability obtained by Pearson's chi-square and Fisher's exact tests