RESEARCH ARTICLE

Prevalence of Aflatoxin Induced p53 Mutation at Codon 249 (R249s) in Hepatocellular Carcinoma Patients with and without Hepatitis B Surface Antigen (HBsAg)

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Abstract

Background: A missense mutation in exon 7 (R249S) of the p53 tumor suppressor gene is characteristic of aflatoxin B1 (AFB1) exposure. AFB1 is believed to have a synergistic effect on hepatitis virus B (HBV) carcinogenesis. However, results of studies comparing R249S prevalence among patients are conflicting. The aim of this study was to determine the prevalence of the R249S mutation in hepatocellular carcinoma (HCC) patients with or without positive HBsAg. Materials and Methods: Paraffin embedded liver tissues were obtained from 124 HCC patients who underwent liver resection and liver biopsy in King Chulalongkorn Memorial Hospital. Restriction fragment length polymorphism (RFLP) was utilized to detect the R249S mutation. Positive results were confirmed by direct sequencing. Results: Sixty four (52%) patients were positive for HBsAg and 18 (15%) were anti-HCV positive. 12 specimens tested positive by RFLP. Ten HCC patients (8.1%) were confirmed to be R249S positive by Sanger sequencing (AGG to AGT). Out of these 10, six were HBsAg positive, and out of the remaining 4, two were anti-HCV positive. The R249S prevalence among HCC patients with positive HBsAg was 9.4% compared to 6.7% for HBsAg negative samples. Patients with the R249S mutation were younger (55±10 *vs* 60±13 year-old) and tended to have a more advanced Edmonson-Steiner grade of HCC, although differences did not reach statistical significance. Conclusions: Our study shows moderate prevalence of aflatoxin B1-related p53 mutation (R249S) in HCC with or without HBsAg. HBsAg positive status was not associated with R249S prevalence.

Keywords: Aflatoxin B1 - R249S - p53 mutation - 249ser - Hepatocellular carcinoma - HBsAg

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Introduction

Aflatoxin is carcinogenic and associated with Hepatocellular carcinoma (HCC) (Yoo, 2010). HCC is a common cancer worldwide and especially in the Asia-pacific region (Srivatanakul et al., 2004). Cirrhosis from chronic hepatitis B and C, alcohol induced liver disease and non-alcoholic steatohepatitis are common risk factors for HCC. Aflatoxin B1 (AFB1) exposure is also considered a risk factor for HCC (El-Serag and Rudolph, 2007; Williams et al., 2004).

Mutation at the third base pair of codon 249 of exon 7 of the p53 gene from guanine to thymine (R249S) is thought to be specifically correlated to AFB1 exposure. This missense mutation is the most common p53 mutation detected in HCC patients from high AFB1 exposure areas (Bressac et al., 1991; Hsu et al., 1991; Yang et al., 1997). Aflatoxin exposure can induce the equivalent mutation in an in-vitro liver cell culture model (Smela et al., 2002; Besaratinia et al., 2009; Gouas et al., 2010). Previous studies have shown that the R249S mutation occurs almost exclusively in HCC and is extremely rare in other cancers. For example, a large number of lung cancers and breast cancer tissues were tested for p53 tumor suppressor gene mutations, and none of them were the aflatoxin-specific R249S mutation (Behn et al., 1998; Cuny et al., 2000).

Several in vitro studies have found synergistic effects between HBV infection and aflatoxin exposure on the TP53 mutation (Qu et al., 2005). In addition, mutation in the X protein of HBV (HBx) has also been correlated with R249S-related carcinogenesis (Jiang et al., 2010; Ortiz-Cuaran et al., 2013). Prospective cohort and nested case-control studies from China and Taiwan (Ross et al., 1992; Wang et al., 1996; Wu et al., 2009) indicate a

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multiplicative risk of developing HCC in patients with concomitant aflatoxin exposure and HBV infection. For these reasons, HBV vaccination has been proposed as a means to prevent aflatoxin-induced HCC (Khlangwiset and Wu, 2010).

However, the result from a meta-analysis did not conclude whether HBV is associated with increased R249S mutation rate (Stern et al., 2001). Out of 15 studies analyzed from the US (Hoque et al., 1999), Europe (Volkmann et al., 1994) and Asia, only 1 R249S mutation was documented in patients without HBV infection. Furthermore, data from developing countries with moderate aflatoxin exposure showed similarly low rates of the mutation (3 R249S mutations from 11 studies) (Buetow et al., 1992; Soini et al., 1996). However, although the number of patients included in meta-analysis was large, the study population and method of R249S testing were heterogeneous.

Recent studies from non HBV endemic areas have demonstrated significant R249S mutation rates in HCV-related HCC and non-HCV-related HCC (El-Kafrawy et al., 2005; Kirk et al., 2005; Nogueira et al., 2009). However, the number of HBV-related HCC patients was too low for a statistical comparison between HBsAg positive and negative subgroups. A more recent study in Thailand demonstrated that the R249S mutation was more common in HBsAg positive group: The R249S mutation was identified in 19 of 48 (40%) HBsAg positive HCC patients and 9 of 35 (26%) HBsAg negative HCC patients. However, this study also lacked an adequate number of cases to make statistical conclusions (Villar et al., 2012). Thus, although the risk of HCC attributed to aflatoxin in Thai HBsAg-positive patients is estimated to be 30-fold more than HBsAg negative individuals (15.9-21.9 versus 0.53-073/10,000 person-years) (Liu and Wu, 2010) no previous study has been conclusive. Therefore, the aim of this study is to determine if a statistical association exists between HBV surface antigen (HBsAg) status and R249S mutation status in HCC patients using a patient population of adequate sample size.

Materials and Methods

Patients

Patients with pathologically confirmed HCC were identified using a computerized database. Surgical specimens were obtained from patients that had undergone hepatic resection and percutaneous liver biopsy in King Chulalongkorn Memorial Hospital, Bangkok, Thailand. HBsAg, alpha-fetoprotein (AFP) and other serology tests were extracted from patients' records. Pathological data including diagnosis, grade, pattern and tumor differentiation were reported by a specialized pathologist. All patients and respective specimens were anonymous. The protocol of the study was approved by the ethical committee, faculty of Medicine, Chulalongkorn University (IRB No. 496/52)

Inclusion criteria consisted of pathologically proven HCC along with available HBsAg results. Exclusion criteria included unavailable paraffin embedded tissue, secondary liver tumors and/or a concomitant cancer diagnosis.

Tissue samples and preparation of DNA

The materials for genetic studies was extracted from paraffin embedded tissue from patients with HCC and were retrieved from the department of pathology, faculty of medicine, Chulalongkorn University. DNA was extracted using a commercial DNA extraction kit (5 prime GmbH, Hamburg, Germany) according to the protocol provided by the manufacturer. The extracted DNA was eluted in 50 μl of distilled water and stored at -200C until polymerase chain reaction (PCR) was run.

PCR amplification and restriction enzyme digestion

Nested PCR was used to amplify the DNA region of interest. The first round PCR mixture contained 2 μl of DNA template, 10 μl of PerfectTaq Plus MasterMix Kit (5 prime GmbH, Hamburg, Germany) 1.25 mM of the outer forward primer (TP53-OS: 5'-CTT GCC ACA GGT CTC CCC AA -3'), 1.25 mM of the outer reverse primer (TP53-OAS: 5'-AGG GGT CAG CGG CAA GCA GA-3') and distilled water (DW) to a final volume of 25 μl. The first round PCR program was the following: 94^{o}C for 3 minute, 40 cycle of 94^{o}C for 18 second, 50^{o}C for 21 second, and 72^{o}C for 1.30 minute concluded by 72^{o}C for 10 minute. The PCR mixture and PCR program of the second round were the same as those of the first round except for the following: an inner forward primer (TP53-OS: 5'-AGG CGC ACT GGC CTC ATC TT-3') and innner reverse primer (TP53-OAS: 5'-TGT GCA GGG TGG CAA GTG GC-3') were used and 0.5 μl of first round PCR product was used as the template for the second round of PCR. The amplification result was then run on a 2% agarose gel and visualize by UV transluminator after staining with ethidium bromide. The TP53 mutation was analyzed by a modified version of restriction fragment length polymorphism (RFLP) as described by Szymanska (Szymanska et al., 2004). Briefly, second round PCR products were subjected to digestion with the restriction endonuclease, HaeIII, which recognizes the sequence CCGG that encompasses the 249 codon. The RFLP mixture contained 1 unit of HeaIII (New England BioLabs inc, Ipswich, MA), 2 μl of 10X Buffer 4 (New England BioLabs inc, Ipswich, MA), 15 μl of second round PCR product and distilled water to a final volume of 20 μl. This mixture was then incubated at 37^{o}C overnight and subsequently run on a 3% agarose gel. RFLP should cut wild-type DNA at three sites thus producing three bands (12, 61, and 92 bp), whereas RFLP should only cut and produce two bands in the mutant (12 and 153 bp). Specimens with positive RFLP for R249S were then confirmed by Sanger sequencing.

Statistical analysis

An adequate sample size to reach statistical significance was determined based an estimated mutation rate of 30% for HBsAg positive samples and 10% for HBsAg negative samples (Dupont and Plummer, 1990). It was determined that at least sixty-two cases and 62 controls were required

Results are expressed as mean±SD. The difference in the R249S mutation rate between subgroups was

determined by a chi-square test. Statistical significance was established as a P value of less than 0.05. Calculations were performed using SPSS version 17.0 (SPSS, Inc., Chicago, Ill)

Results

Patients' characteristics, yield of DNA extraction and RFLP

169 formalin fixed paraffin embedded tissue blocks were included in the study. Their source was from liver resection or liver biopsy specimens collected between 2007 and 2010 that were labeled as HCC according to the pathology department. Eight cases were excluded due to the unavailability of HBsAg results (2 cases) and the final pathological diagnosis not being HCC (6 cases). In addition, 13 pairs of samples belonged to the same patients. Thus, 148 samples were eligible for the study.

DNA could be successfully extracted and tested for the R249S mutation by RFLP in 124 HCC cases. DNA extraction or RFLP processes failed on 24 specimens, most of which were obtained by percutaneous liver biopsies. Sixty-four of the 124 cases (51.6%) were positive for HBsAg. Eighteen patients (14.5%) were positive for HCV antibody. Three cases were positive for both HBV and HCV. The median AFP level was 20.1 IU/ml. Details are described in Table 1.

R249S mutation rate determined by RFLP and confirmed by sequencing

RFLP revealed the R249S mutation in 12 out of the 124 specimens. Those 12 specimens were sent for sequencing and revealed AGG to AGT missense mutation (R249S) in 10 specimens (8.1%) and a silent mutation (AGA) in 2 specimens. No R249S mutation was detected in 10 control liver tissue specimens without HCC.

Prevalence of R249S mutation in HCC with versus without HBsAg

R249S mutation was found in 9.4% (6 of 64) of HBsAg positive HCC specimens versus 6.7% (4 of 60) of HBsAg negative HCC patients. Categorized by serologic etiology, R249 prevalence was 9.8% (6/61) in HBV-related HCC specimens, 13.3% (2/15) of HCV-related HCC specimens, 4.4% (2/45) of non-B non-C HCC specimens and 0%(0/3) of HBV-HCV co-infected HCC specimens. HBsAg status was not significantly associated with R249S mutation (p=0.58).

Table 1. Characteristics of HCC Patients Included in the Study

Study population		(n=124)
Male (%)		103 (83)
Mean age, year		59.3±12.7
HBsAg positive (%)		64 (51.6)
Anti-HCV positive (%)		18 (14.5)
Liver biopsy specimen (%)		38 (30.6)
Tumor differentiation	-well differentiated %	25
	-moderately differentiated %	55
	-poorly differentiated %	20
Tumor pattern	-mixed (%)	48 (39)
	-trabecular (%)	51 (41)
Mean AFP (IU/ml)		6004±26811
Median AFP (IU/ml)		20.1
R249S mutation (%)		10 (8.1)

*Character (% of R249S mutation among those subgroup)

Table 2. Comparison between Characteristics of HCC Patients with Mutant Versus Patients with Wild Type Codon 249

Character (% with mutation)	R249S Mutant (n=10)	Wild type (n=114)	p value
Male (%)	9 (89 %)	94	>0.05
Mean age, year	55±10	60±13	>0.05
Serology			
HBsAg positive	6 (9.4)	58	>0.05
Anti-HCV positive*	2 (11.1)	16	>0.05
Etiology by serology (%)			
HBV-HCV coinfection	0 (0)	3	
HBV (HBsAg)	6 (9.8)	55	
HCV (anti-HCV)	2 (13.3)	13	
Non B Non C	2 (4.4)	43	
Tumor differentiation			
Well	2 (6.7)	28	>0.05
Moderately	5 (7.6)	61	>0.05
Poorly	3 (12.0)	22	>0.05
Tumor pattern			
Mixed (%)	4 (8.3)	44	>0.05
Trabecular (%)	4 (7.8)	47	
Mean AFP (IU/ml)	2486±7828	6327±27911	>0.05
Median AFP (IU/ml)	9.1	23.7	
Log AFP	1.2±1.2	1.67±1.4	>0.05
Mean CA 19-9	99.6±14.3	39.7±19.7	>0.05
Cirrhosis by pathology (%)	4 (10.3)	35	>0.05
Liver biopsy specimen (%)	2 (5.3)	36	>0.05
Positive tumor at margin of resection (%)	1 (11.1)	8	>0.05

*Anti-HCV serology were available for 108 cases

Prevalence of R249S mutation and other variables

Patients with R249S mutation were younger (55±10 *vs* 60±13 year-old) and tended to have a more advanced Edmonson-Steiner grade of HCC, though differences did not reach statistical significance.

Discussion

The study objective was to compare the differences R249S mutation rate between HBsAg positive and negative patients. Our results demonstrate that HBsAg status was not statistically related to an increased or decreased rate of R249S mutation. Studies from Brazil (Nogueira et al., 2009) and Egypt have also reached similar conclusions (El-Kafrawy et al., 2005). These results continue to suggest the importance of decreasing aflatoxin contamination and exposure in the general population regardless of HBsAg status (Nogueira et al., 2009; Liu and Wu, 2010; Hamid et al., 2013).

In this study, the prevalence of the R249S mutation in Thailand was found to be 8.1%. In addition, the average Thai person's exposure to aflatoxin contamination is 53 ng/kg body weight/day aflatoxin B1 intakes. Thus, although universal Hepatitis B vaccine programs can decrease incidence of HCC (Wichajarn et al., 2008) - with both immune memory and antibody persisting for up to 20 years (Poovorawan et al., 2012) - aflatoxin exposure may have still made significant contributions to some hepatocarcinogenesis. We therefore believe that the

reduction of aflatoxin intake in the Asia pacific region is a sensible policy to help mitigate risk of HCC (Bridges et al., 2011).

However, besides peanuts, corn and red chili are both common sources of aflatoxin exposure and these foods are difficult to avoid in the Asia pacific region. Moreover, without access to these foods nutritional status may suffer and it has been shown that good nutritional status in chronic liver disease is associated with improved outcome (O'Brien and Williams, 2008). Thus a recommendation to avoid specific foods such as peanuts and corn seem like an impractical suggestion.

The R249S mutation rate reported in our study and our recently report (Thongbai et al., 2013) are lower than those reported in a previous Thai study. The explanations may be the following: *i*) Compared to past levels, (Sripathomswat and Thasnakorn, 1981), aflatoxin contamination may be lower due to improved food storage and processing (Waenlor and Wiwanitkit, 2003); *ii*) Foods in Bangkok and the central part of Thailand may have lower concentration of aflatoxin than the northern part of Thailand where the previous study was conducted; *iii*) HCC patients from northern Thailand likely have a different genetic background compared to patients from central Thailand and Bangkok, and genetics may play a role in aflatoxin related carcinogenesis (Kirk et al., 2005). However, the difference in the R249S detection method is likely not an explanation for the lower prevalence reported in our study. The previous study used Short Oligonuceotide Mass Analysis (SOMA) to determine R249S prevalence in patients, and this method has been demonstrated to be 1.40 fold more sensitive than RFLP in discovering R249S mutations in a sample population (Qian et al., 2002). Thus, even if the R249S prevalence was 1.40 fold greater than what we found in our study, the prevalence would still only be 11.3%, which is a considerably lower value than what the previous study in Thailand reported. Finally, a limitation of our study may be the use of paraffin embedded tissue because paraffin preservation may fragment or denature DNA strands.

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