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p53 Mutation in Hepatocellular Carcinoma in Northern Thailand

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ABSTRACT

Background: A specific mutation in the p53 gene at codon 249 has been detected in HCC from areas with high exposure to dietary contaminated with aflatoxin B1 (AFB1) , whereas this mutation is absent from HCC in regions with negligible exposure to AFB1.

Objective: The aim of our study was to examine p53 codon 249 mutation in HCC tissue from patients resided in Northern Thailand, and analyzed the relationship between this mutation and clinicopathologic factors.

Patients and Methods: Between January 2001 to June 2003, total 25 liver samples were collected from HCC patients. Mutation of the p53 gene at codon 249 was detected by SOMA.

Results: Of 25 cases, there were 21 samples (84%) with p53 gene expression, and 4 samples were undetectable (16%). Mutation at codon 249 of p53 gene was detected in 6 samples (24%), and wild-type p53 was found in 15 samples (60%). There was no statistically significant association between p53 mutation at codon 249 and age, HBV infection, HCV infection, Child class, tumor size, or AFP level. Only male sex had correlation with this mutation. ($p < 0.05$)

Conclusion: HCC from Northern Thailand has high prevalence of p53 codon 249 mutation compare to other countries with high incidence of aflatoxin exposure. Further studies in non HCC patients as well as the analysis of aflatoxin contamination in food product in Thailand is needed.

Key words : p53 codon 249 mutation, aflatoxin B1, HCC

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BACKGROUND

Hepatocellular carcinoma (HCC), one of the major malignant neoplasm in the world, is the leading cause of cancer related death[1,2]. There is a striking geographical variation in incidence. Both viral and chemical carcinogen are involved in multistage process of human hepatocarcinogenesis[2]. The chronic hepatitis B infection has been well documented as one of the most important risk factor of HCC. In addition to chronic HBV infection, there are also other several factors including chronic hepatitis C infection, alcoholic cirrhosis and cirrhosis from other causes. Aflatoxin, a group of mycotoxins produced by the common fungi *Aspergillus flavus* and *Aspergillus parasiticus*, are established human hepatocarcinogens. Individual susceptibility to aflatoxin-induced hepatocarcinogenesis may be modulated by both genetic and environment factors affecting metabolism[2-5].

Mutations in the p53 tumor-suppressor gene have been identified in the majority of human cancers, and distinct mutational spectra are observed within this gene across cancers of different tissues. The most striking example of a specific mutation in the p53 gene is a G → T transversion in the third base of codon 249, which has been detected in 10-70% of HCC from areas with high exposure to dietary contaminated with aflatoxin-B_1 (AFB_1), whereas this mutation is absent from HCC in regions with negligible exposure to AFB_1[8-13]. Humans are exposed to AFB1 by eating contaminated rice, corn, peanuts, or products of animals that have ingested contaminated food[8].

In Thailand, HCC is one of the most common cancers. Etiology factors that have been associated with the development of the disease include infection with hepatitis B or hepatitis C and dietary exposure to AFB_1. However, the precise mechanism underlying the development of HCC is still not clear. Detection of p53 mutation and it's gene expression have been extensively studies in HCC from a wide geographic around the world[3,5,11,13-21], but the presence of p53 mutations in HCC from Northern Thailand has not yet been reported. In this study, we examined p53 gene mutation at codon 249 in HCC tissue from patients resided in Northern Thailand, using an electrospray ionization mass spectrometry (ESI-MS) based method called short oligonucleotide mass analysis (SOMA). We also analyzed the relationship of p53 mutation at codon 249 with the clinicopathologic factors.

PATIENTS AND METHODS

Patients

The liver samples were taken from HCC patients who were admitted to Maharaj Chiang Mai Hospital from January 2001 to June 2003. Eligible patients were HCC men and women aged 15 years and older whom liver biopsy were taking for diagnosis of liver mass or those required surgical tumor resection. All samples were sent to pathologists for histological diagnosis of HCC, and at least 2 pathologists confirmed the presence of HCC. Tissue sample must be adequate for DNA extraction and further analysis. The liver samples were then processed into paraffin-embedded block and cut into 10 μm-think for DNA Extraction.

Patients' data The data of all 25 patients were reviewed from the hospital charts including age, sex, inhabitant, occupation, alcohol consumption, and laboratory data of complete blood count (CBC), liver function test (albumin, alanine aminotransferase, total bilirubin), prothrombin time, hepatitis marker for hepatitis B virus (HBsAg) and hepatitis C virus (anti-HCV), alpha-fetoprotein (AFP) level, and imaging study to define tumor size. Child-Pugh score was classified from the data of all patients.

Methods

DNA Extraction Genomic DNA was prepared from paraffin-embedded tissue using a Pinpoints Slide DNA Isolations System™ (Zymo Research, Orange, CA) according to the manufacturer's recommendations. A final elution volume of 10 μL was used.

PCR Primers used for PCR amplification were as follow:

(a) p538F1: 5' CTACAACTACATGTGTAAC AGCTGGAGCATGGGCGGCATGAAC-3'; and

(b) p53-8R1: 5' -CTGGAGTCTTCCACTGG AGTGATGGTGAGGATG-3'

Reactions were performed with 2 μL of DNA eluate from tissue samples. The final reaction volume was 50 μL. Negative control (no DNA added) was included for each set of PCR reactions. The PCR products were run on agarose gel.

Mutation Detection by SOMA: SOMA was performed as described by Jackson *et al*[22]. The PCR products were purified by ethanol precipitation and digested with 8 units of BpMI to release 8-bp internal fragments. A phenol-chloroform extraction followed by an etha-

nol precipitation in the presence of See DNA (Amersham Pharmacia, Piscataway, NJ) was performed to purified samples for analysis by ESI-MS. Mass spectra were obtanied with a LCQ Deca ion-trap mass spectrometer (ThermoFinnigan Corp., San Jose, CA), and were programmed to acquire data using scan events monitoring each oligonucleotide individually. A samples was considered positive when fragments were observed in either or both sense and antisense channels for the mutant allele in at least 3 scans across the peak.

Statistical Analysis

Data were analyzed using SPSS version 10.0 (SPSS Inc., Chicago, IL). The p53 was categorized into 3 groups; wild-type, mutation at codon 249,and not detected. The age is expressed as median (range). Pearson's chi sqaure and Fisher's exact test were used to assess the relationship between sex, HBV marker, Child class, AFD level, tumor size and p53 expression where appropriate. The p value of less than 0.05 was considered as statistically significant.

RESULTS

During the time period there were total 45 cases of HCC, and 25 case with adequate tissue samples were included. Of these 25 cases, there were 21 samples (84%) with p53 gene expression, and 4 samples were undetectable (16%). Mutation at codon 249 of p53 gene was detected in 6 samples (24%), and wild-type p53 was found in 15 samples (60%).

Clinical and laboratory data for the patients were listed in Table 1. The relationship between p53 gene expression and clinicopathological features was summarized in Table 2. There was no statistically significant association between p53 mutation at codon 249 and age, HBV infection, HCV infection, Child class,

Table 1 Clinical and laboratory data of 25 patients with hepatocellular carcinoma

Number	Sex	Age	AFP	HBsAg	Anti HCV	Child Class	Tumor Size*	p53+
1.	F	70	45.3	positive	negative	A	large	wt
2.	F	52	10.8	positive	negative	A	diffuse	wt
3.	M	48	133.4	negative	positive	A	0.7	nd
4.	M	62	105.9	positive	positive	A	7	wt
5.	M	63	74.3	negative	positive	A	8.3	wt
6.	M	40	103.4	positive	negative	B	large	mu
7.	M	74	337.9	negative	negative	A	11	wt
8.	M	58	67.3	positive	negative	A	large	wt
9.	F	38	1.3	negative	negative	A	13	wt
10.	M	58	3.9	positive	negative	A	10	wt
11.	F	26	>10,000	positive	negative	A	large	wt
12.	M	47	18.8	negative	positive	A	13	mu
13.	M	72	1.9	positive	negative	A	diffuse	nd
14.	M	53	12.3	positive	negative	B	diffuse	mu
15.	F	28	1.1	negative	negative	A	10	wt
16.	M	60	29.4	positive	negative	A	3	mu
17.	M	51	3.8	positive	negative	A	2	mu
18.	M	57	22.3	positive	negative	B	3.9	wt
19.	M	66	2.2	positive	negative	A	6.9	wt
20.	F	74	2	negative	negative	A	8	wt
21.	F	68	280	positive	negative	C	6.5	wt
22.	M	50	93.2	positive	negative	A	diffuse	mu
23.	M	45	313.4	negative	positive	A	large	nd
24.	M	60	4.2	positive	negative	B	1.2	wt
25.	M	38	>10,000	positive	negative	B	diffuse	nd

**Diameter of tumor; large >15 cm, diffuse = infiltrative type*

+*wt = wild-type, mu = mutaton at codon 249, nd = not detect*

Table 2 Association of p53 protein with clinicopathological data of HCC from Northern of Thailand

Characteristics	No. of cases (n = 25)	p53%			p values
		Wt (n = 15)	Mu (n = 6)	Nd (n = 4)	
Sex					
Male	18	8 (44.4)	6 (33.3)	4 (22.2)	
Female	7	7 (100)	0	0	0.03
Age (years)					
Median	25	58 (26-74)	56 (40-60)	58 (38-72)	NS
HBV marker					
HBsAg (+)	17	10 (58.8)	5 (29.4)	2 (11.8)	
HBsAg (-)	8	5 (62.5)	1 (12.5)	2 (25)	NS
HCV marker					
Anti HCV (+)	5	2 (40)	1 (20)	2 (40)	
Anti HCV (-)	20	13 (65)	5 (25)	2 (10)	NS
Child Class					
A	19	12 (63.2)	4 (21)	3 (15.8)	
B	5	2 (40)	2 (40)	1 (20)	
C	1	1 (100)	0	0	NS
Tumor size					
<5 cm	5	2 (40)	2 (40)	1 (20)	
5-10 cm	7	7 (100)	0	0	
>10 cm	13	6 (46.1)	4 (30.8)	3 (23.1)	NS
AFP					
<400	2	1 (50)	0	1 (50)	
>400	23	14 (60.9)	6 (26.1)	3 (13)	NS

Ns = not significant

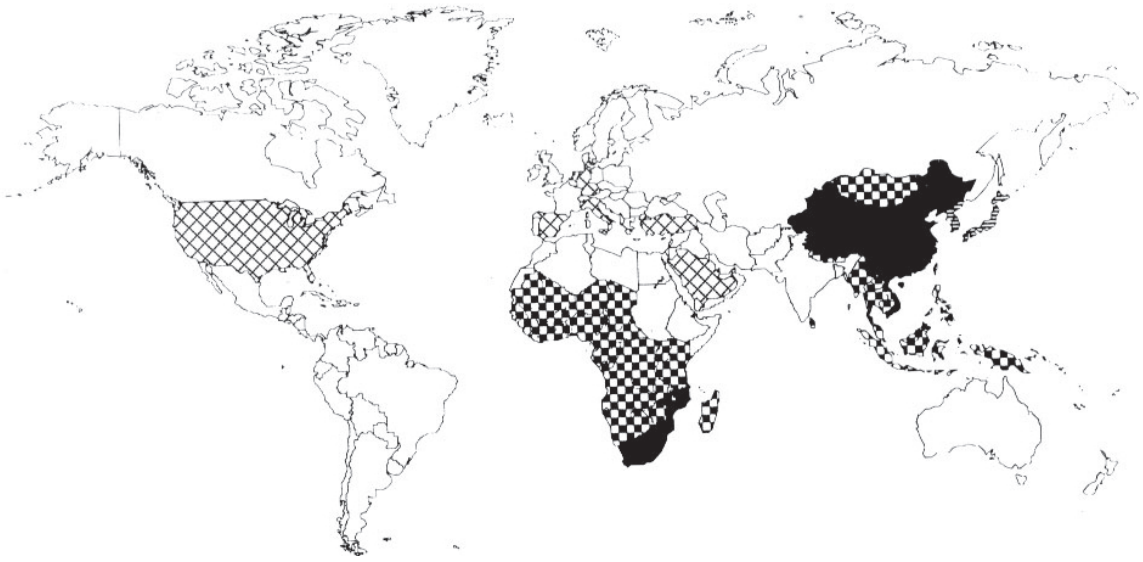

Figure 1 p53 gene codon 249 mutation and the global incidence of hepatocellular carcinoma, (reproduced from M. Ozturk[24])

tumor size, or AFP level. However, there was a good correlation between sex and p53 mutation at codon 249. Among 25 cases, most of them was male (72%). Mutation at p53 codon 249 was detected only in males ($p < 0.05$). All 7 female patients had wild-type p53 expression while there were 8 out of 18 (44.4%) in males. The mediam ages of cases with wild-type p53, mutation at codon 249 and undetectable were 58,56,58 respectively. Viral marker of HBV, HBsAg, and HCV, anti-HCV, were positive in 66.7%, 13.3% of wild-type p53 group and 83.3%, 16.7% of mutation group. Most of patients in each group were in Child A, and had large tumor size of more than 10 cm. Diameter. Alpha-fetoprotein level was not high; defined as less than 400, in almost all patients of three groups.

DISCUSSION

HCC is a very prevalent from of cancer in the world. Wide variation of HCC incidence in different areas of the world suggests the involvement of environmental factors in its etiology. Available evidence clearly establish HBV as a major risk factor for HCC. However, within those high incidence areas, there is heterogeneity in clinical, pathological, and causal aspects of the condition as well as in the incidence of HCC. Environmental factors that putatively contribute to the etiology of HCC include aflatoxin contamination of the diet, ingestion of alcohol beverage, consumption of diets low in selenium, tobacco smoking, and androgen therapy[23]. On the basis of molecular studies, the presence of specific p53 codon 249 mutational hotspot correlates with aflatoxin exposure. M. Ozturk[24], described the mutation of p53 gene codon 249 and global incidence of HCC. He showed that China has a high incidence of HCC with evidence of p53-249 mutation, and also Southern Africa and Vietnam (Figure 1). Unit recently, the frequency of p53 mutation at codon 249 in HCC has been reported differently in various geographic areas: 10-57% in China (Qidong, Guangxi, Harbin, Tongan, Fusui)[11-19,24,25], 4-26% in South Africa[24-27], 13% in Taiwan[5,6], 33% in Vietnam[24] while undetectable in Korea, Japan and USA[19]. In our study, we showed that p53 codon 249 mutation in HCC in population of Northern Thailand was 24%. This result was in the same range of mutation found in tumors from China, South Africa and Vietnam where was the high exposure area to aflatoxin. Although we did not have the data of the dose of AFB_1 contaminated in food product from Thailand, one may assume high contamination like other countries in Southeast Asia[28,29] because of the similar climate and dietary habit.

Clinicopathologic factors that associated with p53 codon 249 mutation had been study only in Guangxi, Southern China[25]. Age under 40 years, large tumor size and tumor differentiation were correlated to this mutation. Contrasted to our study, we found that only male sex was associated with p53 mutation at codon 249. Although HCC was seen more in male, we cannot find good explanation for this association. One suggestion was interaction with alcohol consumption, which seen more frequent among males. But unfortunately, alcohol consumption was not report in the results because we did not know the exact amount of alcohol taken by the patients.

For other factors, we could not demonstrate their association with p53 mutation. The main reason for this was that we had rather small sample size. In addition, this study collected data of the patients retrospectively from the hospital charts. So the data were incomplete in several aspects. Moreover, the cases in this study were a selective group. Most of samples from liver biopsies were taken for the diagnosis of liver mass when AFP level was less than 400 ng/ml. So we did not include several HCC patients whose AFP levels were greater than 400 ng/ml, whom liver biopsy were not necessary for the diagnosis. It is also noted that bnearly the same number of patients was excluded because the tissue samples were not adequate for DNA extraction.

Several previous studies have demonstrated that p53 mutation was detected in corresponding with HBV infection[2,5,8,10,18,30,31], while other reports have indicated no significant difference between HCC with or without HBV infection[17,24,25]. In this study, we were also unable to demonstrate a good relationship between HBV infection and p53 gene mutation. However, we did find that 83.3% of the patients with p53 codon 249 mutation show positive HBsAg.

A specific hotspot mutation at codon 249 of the p53 gene with G to T transversion is frequently detected in HCC from area with high exposure to AFB_1, where this mutation is absent from HCC in regions with negligible exposure to AFB_1. This study provides further support for the role of aflatoxin exposure and HCC in Northern Thailand where the mutation of p53 codon 249 is found to be comparable with other countries with

high incidence. The role of HBV infection as synergistic effect to AFB_1 has not been concluded in this time. Further studies is needed to determine the mutation of codon 249 of the p53 gene in non HCC with or without HBV infection compare to HCC patients as well as the analysis of aflatoxin contamination in food products in Thailand.

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