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• LIVER CANCER •

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

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Abstract

AIM: One of the characteristics of hepatocellular carcinoma (HCC) in Qidong area is the selective mutation resulting in a serine substitution at codon 249 of the p53 gene (1, 20), and it has been identified as a "hotspot" mutation in heptocellular carcinomas occurring in populations exposed to aflatoxin and with high prevalence of hepatitis B virus carriers (2, 3, 9, 10, 16, 24). We evaluated in this paper whether this "hotspot" mutation could be detected in cell-free DNA circulating in plasma of patients with hepatocellular carcinoma and cirrhosis in Qidong, China, and tried to illustrate the significance of the detection of this molecular biomarker.

METHODS: We collected blood samples from 25 hepatocellular carcinoma patients, 20 cirrhotic patients and 30 healthy controls in Qidong area. DNA was extracted and purified from 200 μl of plasma from each sample. The 249^{Ser} p53 mutation was detected by restriction digestion analysis and direct sequencing of exon-7 PCR products.

RESULTS: We found in exon 7 of p53 gene G→T transversion at the third base of codon 249 resulting 249^{Arg}→249^{Ser} mutation in 10/25 (40 %) hepatocellular carcinoma cases, 4/20 (20 %) cirrhotics, and 2/30 (7 %) healthy controls. The adjusted odds ratio for having the mutation was 22.1 (95 % CI, 3.2~91.7) for HCC cases compared to controls.

CONCLUSION: These data show that the 249^{Ser} p53 mutation in plasma is strongly associated with hepatocellular carcinoma in Qidong patients. We found this mutation was also detected, although it was at a much lower frequency, in plasma DNA of Qidong cirrhotics and healthy controls; We consider that these findings, together with the usual method of HCC diagnosis, will give more information in early diagnosis of HCC, and 249^{Ser} p53 mutation should be developed to a new early diagnostic marker for HCC.

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INTRODUCTION

Qidong is a high risk area of hepatocellular carcinoma (HCC), chiefly due to chronic hepatitis B virus (HBV) infection, and exposure to AFB1[1, 2]. HCC is a major cause of cancer death in this area. Epidemiological and experimental evidence show that hepatitis B virus (HBV) and dietary exposure to aflatoxin B1 (AFB1) contribute to hepatocarcinogensis[3-9]. About 10-15 % of the Qidong populations are chronically infected with HBV (LU. PX *et al*, 1991). An analysis of individual biomarkers of aflatoxin exposure in Qidong (Daxing country) has shown 99 % (791/792) of individuals with detectable serum aflatoxin-albumin adducts (Zhu YR *et al*, 1999).

p53 mutations have been identified in several human cancers[10-12]. A selective G to T transversion mutation at codon 249 (AGG→AGT, arginine to serine) of the p53 gene has been identified as a "hotspot" mutation for HCC in Qidong area[13-16]. Data from Qidong Liver Cancer Institute have suggested that this mutation in HCC is strongly associated with exposure to AFB1[13]. Recent research in Gambia[17] has shown that in some human cancers, circulating tumor DNA can be successfully retrieved from plasma or serum and used as a surrogate material to analyze for genetic alterations present in the original tumor[18-20]. We adopted this approach to evaluate the presence of 249^{Ser} p53 mutation in plasma from HCC cases, cirrhotic patients and healthy controls, and this mutation could be regarded as a new biomarker in HCC earlier diagnosis.

MATERIALS AND METHODS

Subjects and specimens

Blood samples from 25 cases of HCC, 20 cirrhotic patients and 30 healthy controls were collected in Qidong Liver Cancer Institute. The definition for 25 cases of HCC included compatible clinical and ultrasonographic findings and serum AFP levels. The ultrasonographic data were most important. 20 Cirrhotic patients were included as an additional referent group for evaluation of factors associated with progression to HCC. The diagnosis of cirrhosis was also based on compatible clinical history and by ultrasonographic method. 30 cases of controls were recruited from the outpatient clinics among individuals with no history or clinical findings suggestive of liver disease, and have the same distributions of age, gender and recruitment site with the HCC and cirrhotic cases.

DNA extraction

Blood samples anticoagulated with EDTA were processed immediately after collection, plasma was transferred to a plain tube and stored at -70 ℃ at Qidong Liver Cancer Institute. AFP and HBV serological testing was performed using standard laboratory kits. A 500 μl aliquot of plasma was shipped in liquid nitrogen to the state key laboratory of genetic engineering, Fudan University (Shanghai), for 249 Ser testing. DNA was extracted from 200 μl of plasma using a QiAamp blood kit (Qiagen Company) according to the manufacturer' s protocol. The purified DNA was eluted from silica column with 50 μl of Nuclease-Free Water.

PCR

Primers used for PCR amplification synthesized by Sangon company (Shanghai) were as follows: p1 (up) (5' -ctt gcc aca ggt ctc ccc aa-3'), p2 (down) (5' -agg ggt cag cgg caa gca ga-3'). The expected size of the product was 254 bp, and this fragment was located in the exon 7 of p53 gene.

Using TaKaRa PCR kit, the 25 μl reaction included 18.3 μl ddH_2O, 2.5 μl 10×buffer, 1 μl dNTPs, 0.5 μl primer 1, 0.5 μl primer 2, 0.2 μl Taq DNA polymerase, 2 μl DNA templates. The thermo-cycling conditions were 94 ℃ for 5 min, and 40 cycles of 94 ℃ for 30 sec, 60 ℃ for 30 sec, 72 ℃ for 30 sec, and finally 72 ℃ for 10 min in a Peltier Thermal Cycler (PTC 200). The amplification products (254 bp) were visualized by staining with ethidium bromide, after electrophoresis on 2 % agarose gel.

Purification of PCR products

To obtain enough amounts of DNA fragments for further testing, 1 μl of PCR product of each sample was picked up as template to have another PCR reaction with the same amplifying condition. The PCR products were then purified with 3S PCR Product Purification Kit (Biocolor Biological Science & Technology Co., Ltd) to eliminate some impurities such as dNTPs, primers, polymerase, and mineral oil.

Mutation detection by restriction analysis

The 254 bp of purified DNA fragment, which is derived from exon 7 of p53 gene, was submitted to restriction enzyme *Hae III* (New England Biolabs Company) digestion. The restriction enzyme digestion reaction system was as follows: 1 μl *HaeIII*, 2 μl 10×buffer2, 5 μl DNA fragment, 12 μl ddH_2O (20 μl total volume). These reaction systems were submitted to 37 ℃ water incubation for 4 hours. Enzyme *Hae III* cleaves a GG/CC sequence at codons 249-250, generating 92bp, 66bp and several small fragments from the 254 bp purified DNA product of the PCR reaction. If there is a mutation at codon 249-250 resulting in an uncleaved, 158bp fragment, and this feature will be distinguished from that of normal samples on 2 % agarose gel stained with ethidium bromide. Absence of the band at 254 bp (full-length PCR products) provides a control for complete digestion of the PCR product. In our protocol, we also arrange positive (with 249 mutation) and negative (wild-type) controls. The presence of the uncleaved 158 bp fragment indicates that there are mutations in the corresponding samples, and DNA fragments of these special samples were analyzed by automated DNA sequencing (ABI 377) using BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE company).

Statistical analysis

Pearson' s chi-square and Fisher' s exact test were used to assess statistical significance of frequency tables of independent variables and 249 Ser mutations as a dichotomous variable. Multivariable logistic regression analysis was performed to estimate odds ratios (OR) along with 95 % confidence intervals (CI) to estimate the risk of mutation among the different study groups considering age, gender, recruitment site, and hepatitis B surface antigen status as potential confounders.

RESULTS

The characteristics of the 75 subjects from Qidong were evaluated in the study (Table 1).

HCC cases, cirrhosis cases and controls from Qidong were of similar age and gender distribution. 84 % of HCC cases, 65 % of cirrhotics and 13 % of controls were positive for hepatitis B surface antigen (HBsAg), a marker of chronic infection with HBV (P<0.001 for difference between groups). 80 % of HCC cases, 70 % of cirrhotics and 0 % of controls were positive for serum alpha-fetoprotein positive (AFP>100 ng/ml).

Table 1 Characteristics of study participants from Qidong

	Hepatocellular carcinomas	Cirrhotics	Controls
	(25)	(20)	(30)
Age (average [range], in years)	49 (27-70)	39 (25-65)	45 (19-87)
Number of males (%)	20 (80)	17 (85)	25 (83)
Hepatitis B surface antigen positive (%)	21 (84)	13(65)	4 (13)
Serum alpha-fetoprotein positive (%)	20 (80)	14 (70)	0 (0)

The electrophoresis on 2 % agarose gel shows that the 254 bp specific DNA fragments amplified between p1 and p2 are at the appropriate location according to the DNA molecular weight markers (Figure 1).

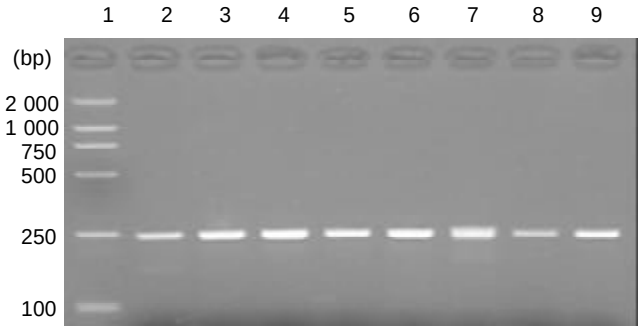

Figure 1 The electrophoresis map of PCR products. Lane 1: DNA Molecular weight markers. Lane 2-9: PCR products of partial samples.

DNA fragment from each sample was digested with restriction enzyme *HaeIII*. Presence of undigested 158 bp fragments on 2 % agarose gel indicates that there is a point mutation in the *Hae III r*ecognition site (Figure 2).

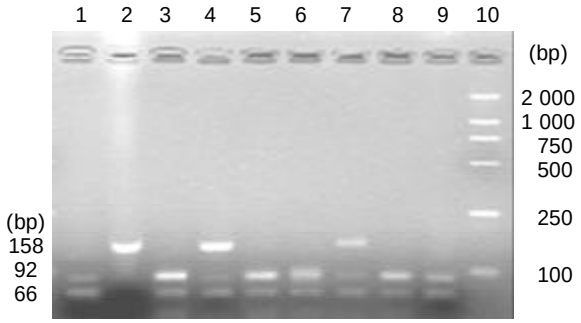

Figure 2 Mutation at coden 249 was identified by restriction digestion. Lane 1: negative control, Lane 2: positive control, Lane 3-9: HCC samples, Lane 10: DNA molecular weight markers.

p53 exon 7 fragments from normal controls (wild type) and mutation (identified by restriction digestion) HCC cases were sequenced (Figure 3).

We found in this paper 16 of 75 subjects from Qidong have 249^{ser} p53 mutation, including ten of 25 HCC cases, four of 20 cirrhotic patients, and two of 30 controls, giving a prevalence of 40 %, 20 % and 7 %, respectively (P<0.001 for difference between groups) (Table 2).

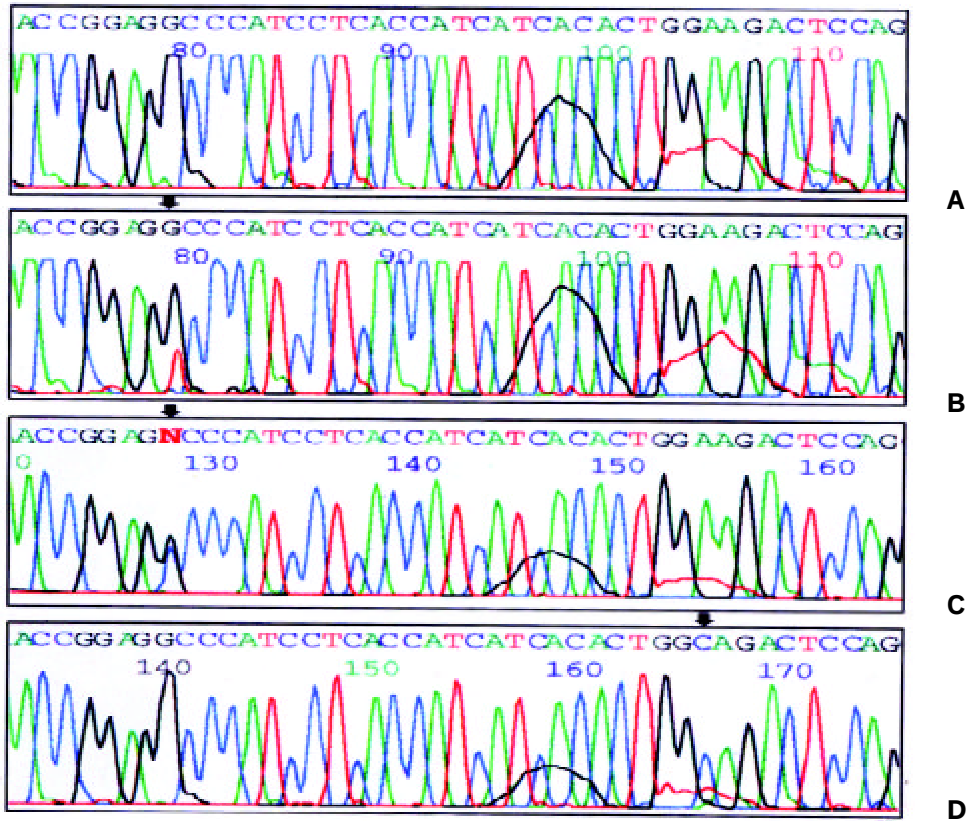

Figure 3 Sequencing results (partial electropherogram). A: Wild type sequence shows codon 249 AGG; B: Mutation sequence shows codon 249 AGG→AGT transversion; C: Mutation sequence shows codon 249 AGG→AGC transversion; D: A→C transversion at the second base of codon 258.

Table 2 Prevalence and adjusted odds ratios for plasma 249 ^{Ser} p53 mutations by subject groups from Qidong

	Controls[a]	Cirrhotics	Hepatocellular carcinomas
	(30)	(20)	(25)
No. positive (%)	2 (7)	4 (20)	10 (40)
OR (95% CI)	1.0	5 (0.6, 29.3)	22.1 (3.2,91.7)

[a]Estimated odds ratios (OR) and 95 % confidence intervals (CI) were adjusted for age, gender, recruitment site and hepatitis B surface antigen status, using controls as the referent group.

DISCUSSION

In the People' s Republic of China, HCC accounts for over 250 000 deaths annually with an incidence rate in some areas of the country approaching 150 cases/100 000/year. Mutations in the p53 tumor suppressor gene have been identified in a majority of human cancers[21], especially in HCC[2,22]. 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 HCCs from area with a high exposure to AFB1[6,23-25], where this mutation is absent from HCC in regions with negligible exposure to AFB1[13,26,27].

Jackson *et al*. reported that a high percentage of the tumors from Qidong had G→T mutation at the third base of codon 249 of the p53 gene than tumors from Shanghai (46.7 % compared with 30 %)[10,28]. The mutation frequency corresponds to exposure to aflatoxins because these areas have high and intermediate exposure levels, respectively. A recent report by Kirk *et al*. reported for the first time the detection of codon 249 p53 mutation in the plasma of liver tumor patients from Gambia. DNA circulating in the serum or plasma can be successfully retrieved and used as surrogate material to analyze for genetic alterations present in the original tumor[10,29]. Although the mechanisms accounting the presence of this circulating DNA are uncertain, there is some evidence that the DNA, of up to 21 Kb, are released from the tumor as a glyconucleoprotein complex, and may protect the DNA from degradation by nucleases. Although we don' t understand the relationship between the release of tumor DNA into the plasma and necrosis of the tumor, apoptosis or other selective cellular processes, we considered from our experimental data that plasma or serum may be used as a source of tumor-specific DNA[30,31].

We reported the presence of 249^{Ser} p53 mutations in DNA circulating in the plasma of Qidong population at high risk for HCC, chronic HBV infection and exposure to the carcinogen AFB1. We found in this paper 16 of 75 subjects from Qidong had 249^{ser} p53 mutation, including ten of 25 HCC cases, four of 20 cirrhotic patients, and two of 30 controls (Table 2). It is interesting that we found in one HCC case a G→C transversion at the third base of codon 249 instead of G→T transversion, although both of AGC and AGT coded serine. The 249^{Ser} p53 "hotspot" mutation was detected in 40 % of HCC cases, and a much lower prevalence was observed in cirrhotic patients (20 %) and in controls (7 %) (P<0.001 for difference between groups). Additionally, we found in another HCC case A→C transversion at the second base of codon 258. We checked the exon 7 of p53 gene using NCBI Blast, and found none of the ESTs could support this point mutation. Is it a SNP or a point mutation associated with AFB1 exposure? It is to be studied. Although we didn' t check the 249^{Ser} p53 mutations in the corresponding tissues, we can draw a conclusion from these data that p53 mutation frequency of these different groups provided the strong relationship between cirrhosis and the future development of HCC[32]. This mutation may be regarded as an early detection marker or a prognostic molecular marker in HCC[33,34]. The detection of 249^{Ser} DNA may be useful as a

maker of neoplastic development, and the presence of the mutation in healthy subjects may reflect chronic exposure to high levels of AFB1[35].

Besides the detection of serum aflatoxin-albumin adducts, we can also use plasma 249^{Ser} p53 mutation as a marker of aflatoxin exposure in epidemiological studies. It is expected in Qidong area that the detection of p53 mutation in plasma DNA could be developed into a usual method to estimate the development of HCC, and this mutation could be regarded as an early diagnostic marker of hepatocellular carcinoma.

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