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Frequency of Mutation of Codon 249, Overexpression of *p53*, and Hepatitis B Virus DNA Positivity in Hepatocellular Carcinoma

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Background: In hepatocellular carcinoma (HCC), the frequency of *p53* mutation and the association with hepatitis B virus (HBV) infection varies with geographic locations and risk factors. The aim of this study was to determine the frequency of codon 249 mutation of *p53*, p53 overexpression, and HBV DNA positivity and to observe the relationship between them in Korean HCC.
Methods: We analyzed overexpression of p53 in hepatoma tissue from 17 HCC patients by immunohistochemistry (IHC), specific mutations at the third base position of codon 249 by PCR-restriction fragment length polymorphism (PCR-RFLP) method, and presence of HBV by nested PCR.
Results: Although a point mutation at codon 250 was seen in one (5.8%) of 17 patients, no codon 249 mutations were found in the patient cohort. The p53 protein was overexpressed in 4 (23.5%) of 17 HCCs. PCR for HBV DNA from HCCs showed a positivity rate of 82.4% (14 of 17 specimens).
Conclusion: In HCC of this study, HBV infection was not associated with either 249 mutation or overexpression of p53, and overexpression of p53 protein seemed to be related to other than this mutation. **(Korean J Clin Microbiol 2007;10:84-89)**

Key Words: Hepatocellular carcinoma, Hepatitis B virus, *p53* mutation, p53 overexpression, Immunohistochemistry

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm and the third most common cause of cancer-related death in the world[1]. Chronic HBV and HCV infections attribute to the HCC development in more than 80% of the HCC cases worldwide[2]. Chronic infection with HBV and ingestion of aflatoxin-contaminated foods are considered major risk factors for HCC in South East Asia, China and sub-Saharan African countries[3,4].

The *p53* gene works as a tumor suppressor gene, which is located on the short arm of chromosome 17 that encodes a 53-kDa nuclear phospoprotein that binds DNA and negatively regulates cell division, preventing progression from G1 to S phase[5,6]. It is well known that the inactivating mutation of *p53* is the most common genetic alteration in human cancers including HCC[7]. It has been demonstrated that the most common mutation associated with HCCs is at codon 249 of *p53*, which is causally related to high aflatoxin B1 exposure[8]. Recent reports indicate that HBV and aflatoxin B1 may influence the development of HCC by altering the *p53* gene[3].

The relationship between HCC and overexpression of the mutant *p53* gene has been studied in different geographic regions, but the results are varied[9]. Thus, we studied the frequencies of p53 overexpression, mutation of p53 at codon 249, and HBV DNA positivity in hepatoma tissue to observe the interrelationship between p53 overexpression and HBV in Korea where the people have a limited exposure to dietary aflatoxin.

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MATERIALS AND METHODS

1. Patients and samples

Seventeen cases of HCC were included in this study. All the patients with HCC underwent hepatectomy from January 1996 to December 2000 at the Chosun University Hospital (Gwangju, Korea).

2. Immunohistochemical staining

Four-micron sections were cut from formalin-fixed paraffin embedded tissue blocks and dehydrated and then deparaffinized according to standard procedures. For antigen retrieval, the sections

were heated in a microwave oven at 98℃ for 10 min in 10 mM citrate buffer at pH 6.0. Endogenous peroxidase activity was eliminated by preincubation in hydrogen peroxide followed by washes in tris-buffered saline (TBS). Sections were stained using standard streptavidin-biotin complex immunoperoxidase methods using anti-p53 antibodies (clone DO-7; diluted 1/50; Dako, Carpinteria, California, USA). Anti-p53 antibody was incubated for 1 hour at room temperature. The slides were counterstained with hematoxylin. For positive control, sections were obtained by colon cancer specimen. Only a distinct nuclear staining in tumor cells was determined as positive and percentage of positive cells per whole tumor cells was counted. The specimens with percent positive cells of more than 10 were interpreted as overexpression[10,11].

3. DNA extraction

Neoplastic lesions of hepatectomy tissue were resected from patients and tissue sections were transferred in a 1.5 mL Eppendorf tube. The samples were digested overnight with 20 μL of 20 μg/mL proteinase K (Sigma Chemical Co., St. Louis, MO, USA), 20 μL of 10% SDS in 300 μL of 1.0× TEN Buffer (1 M Tris, 5 M NaCl, 0.5 M EDTA at pH 8.0) at 54°C and mixed by vortexing. An additional 0.1 μL of 5 M NaCl was added in the mixture, and the samples were mixed again by vortexing. The samples were centrifuged at 15,000 rpm for 5 min at 4°C, and the supernatant was transferred in a new Eppendorf tube. A total of 1 mL of chilled 100% ethanol was added and was placed in −20℃ for 1 hr then, the samples were centrifuged at 15,000 rpm for 15 min at 4°C. From the supernatant, DNA was precipitated and dissolved in 50 μL of distilled water.

4. Nested PCR for core promoter region of HBV DNA

Nested PCR was performed as described previously[12]. The first PCR primer pairs for nested PCR were 5'-CTGCCGTTCCGGCCGACCAC-3' and 5'-AGAAAAAACGGAAGACTGAA-3'. The second PCR primer pairs were 5'-CATAAGAGGACTCTTGGACT-3' and 5'-ATTAGGCAGAGGTGAAAAAG-3'.

5. PCR Amplification of *p53*

The exon 7 of *p53* gene was amplified by PCR using forward primer (5'-GTTGGCTCTGACTGTAC-3') and reverse primer (5'-CTGGAGTCTTCCAGTGT-3') according to the procedures described previously[13].

6. RFLP for detection of mutation of *p53* at codon 249

Fifteen microliters of PCR products were digested with 5 IU each of *Hae*III (New England Biolabs, Beverly, MA, USA) and

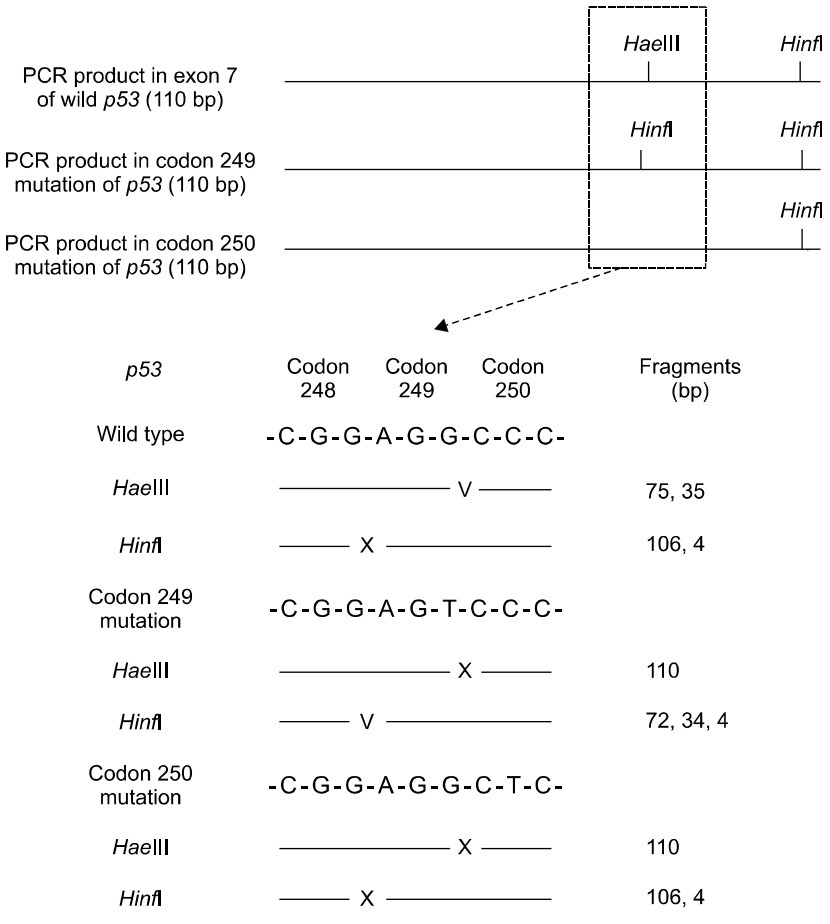

Fig. 1. Illustration of the nucleotide sequence of exon 7 of *p53* gene with relation to *Hae*III and *Hinf*I restriction site. v, cut; x, uncut.

*Hinf*I (New England Biolabs), and the digestion mixtures were incubated at 37°C in a 25 μL volume for 4 hr. Restriction sites for *Hae*III and *Hinf*I are GGCC and GANTC, respectively. As shown in Fig. 1, the PCR product (110 bp) of wild type was cut into 2 fragments of 75 and 35 bp using *Hae*III and into 2 fragments of 106 and 4 bp using *Hinf*I. PCR product carrying the mutation of an AGG to AGT transversion at codon 249 was not cut using *Hae*III, but was cut into 3 fragments of 72, 34, and 4 bp using *Hinf*I, because a *Hinf*I restriction site (GAGTC) was created. If the PCR product carried a mutation at codon 250, it would loose *Hae*III site and would not carry *Hinf*I site. So, PCR product carrying a mutation at codon 250 was not cut using *Hae*III and cut into 2 fragments of 75 and 35 bp using *Hinf*I (Fig. 1).

7. DNA sequencing

The PCR products were purified using JET-SORB gel extraction kit (Genomed, Bad Oeynhausen, Germany) and sequenced using BigDye 2.0 Cycle Terminator kit (Applied Biosystems) and an ABI Prism 377 DNA Sequencer (Applied Biosystems). The sequencing procedure was carried out according to the manufacturer's protocols.

8. Statistical analysis

The statistical analysis was performed using the Statistical Package for Social Sciences for Windows Version 10.0 (SPSS Inc., Chicago, Illinois, USA). Chi-square test was used to assess the association of p53 overexpression with HBV detection in HCC tissue. Two-sided *P* value of less than 0.05 was considered as statistically significant.

RESULTS

1. Overexpression of p53 and positive rate of HBV DNA

p53 immunohistochemistry (IHC) testing revealed overexpression in 4 (23.5%) of 17 patients with HCC. PCR for HBV DNA from hepatoma tissue revealed that 14 (82.4%) of 17 were positive for HBV DNA (Table 1), which included 3 (75%) of 4 p53-overexpressed specimens and 11 of 13 (84.6%) specimens with normal expression of p53. The p53 overexpressionrate in HBV DNA-positive cases was 21.4% (3/14) while that in HBV-negative cases was 33.3% (1/3). No significant association ($P>0.05$) was observed between p53 overexpression and HBV DNA positivity in hepatoma tissue in this cohort of HCC patients.

2. Frequency of *p53* mutation

RFLP patterns did not show any mutations in codon 249 in 17 HCC specimens (Table 1). However, HCC from one of the 17 (5.8%) patients did exhibit both wild and mutant type RFLP patterns (Fig. 2). DNA sequencing of the PCR product of that patient indicated the presence of both a wild type peak and minor mutant

Table 1. Expression and mutation of *p53*, and presence of HBV in hepatoma tissue of 17 specimens of hepatocellular carcinoma

p53 IHC	p53 codon 249 Mutation	p53 codon 250 Mutation	HBV DNA	No. of cases (%)
−	−	−	+	11 (64.7)
−	−	−	−	2 (11.8)
+	−	−	+	2 (11.8)
+	−	−	−	1 (5.9)
+	−	+	+	1 (5.9)

Abbreviation: IHC, immunohistochemistry.

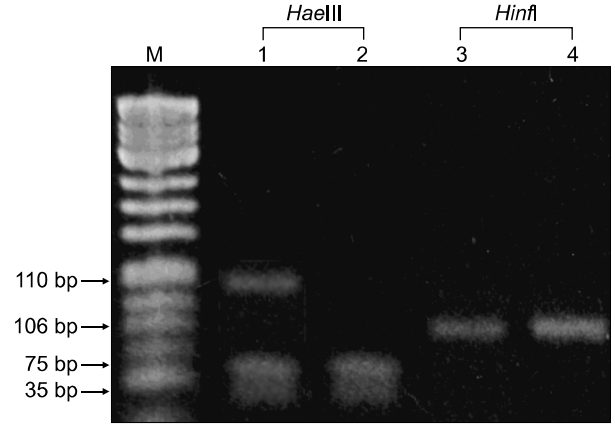

Fig. 2. RFLP pattern of *Hae*III and *Hinf*I digest of exon 7 PCR product of *p53* gene. M, size marker; lane 1 and 3, heterozygote type RFLP pattern that shows wild and codon 250 mutant type RFLP pattern together; lane 2 and 4, wild type RFLP pattern.

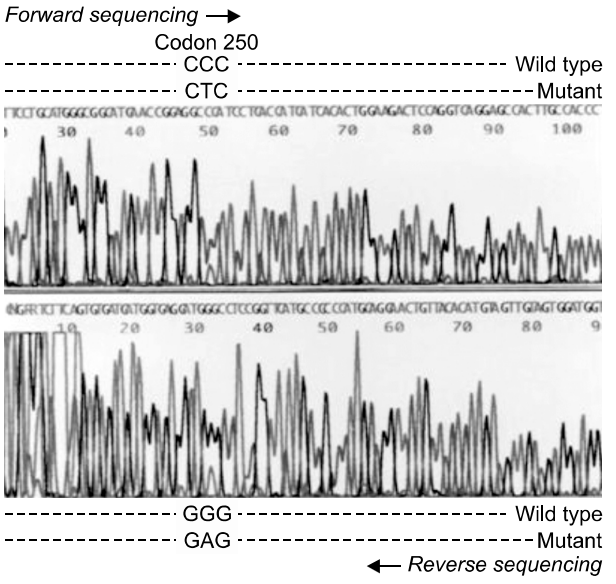

Fig. 3. DNA sequencing analysis of a specimen that shows both wild type & minor mutant peaks in codon 250 of the 7th exon of the *p53* gene.

peak in codon 250 of *p53* (Fig. 3). Forward and reverse sequencing identified a C to T transition of the second nucleotide of codon 250.

DISCUSSION

Immunohistochemical staining revealed overexpression of *p53* in 23.5% (4/17) of the HCCs studied. Similar results were reported in previous studies for HCC patients in Korea[14-16]. In normal state, the p53 protein is continuouslyproduced and degraded in the cell. However, degradation rate of mutant p53 proteins is decreased because theses proteins have less binding efficiency to MDM2 proteins, important negative regulators of p53 protein, than wild type p53 proteins[17]. The fundamental mechanism of p53 overexpression is a mutation of *p53*, although some mutations in the p53 gene can lead to a lack of p53 protein production. Three cases in this study showing overexpression of p53 without 249 mutation could be due to other gene aberrations not analysed in this study, such as mutations of other codon and splicing region and dysfunction of p53 stabilizing system[14].

In our study, one of 17 (5.8%) cases had exon 7 mutation at codon 250, which is similar in frequency to the *p53* exon 7 mutation rate of 4.8% (2/42) observed in HCC[18]. No mutation at codon 249 have yet been found in the Korean population[18,19]. This is consistent with the low prevalence of mutations at codon 249 reported in the geographic areas where high levels of dietary aflatoxin B1 are not detected, including Japan and Korea[19]. Factors other than aflatoxin B1 may be responsible for those *p53* abnormalities observed in Korean HCC. Codon 250 mutation of CCC (Pro) to CTC (Leu) transversion is found in 47 cases including 3 cases of HCC and 44 cases from various tumor specimens in the uncurated *p53* mutation database in all tumors only[20]. Because the codon 250 mutation is commonly found in various tumors, it may be possible that the mutation contribute to tumor progression like other p53 mutations.

Infection with HBV is the most important risk for HCC in Asia except Japan[2]. The high incidence rate of HCC in Korea is associated to the high carrier rate (5～6%) of HBV[21]. Fourteen of the 17 (82.4%) HCC cases were HBV DNA-positive, which is similar to the positivity rate of 68.8～76% reported in previous studies[18,22,23].

In the present study, no significant associations were observed between p53 overexpression and HBV DNA positivity. The relationship between mutation of *p53* with the resultant overexpression of p53 protein and HBV infection in HCC is still controversial. On the one hand, p53 protein could bind the HBx antigen to form complexes, which lead to p53 inactivation[24-27]. Moreover, HBV infection, or the presence of HBV DNA in HCC, is associated with an increased rate of *p53* mutation[28]. On the other hand, some contradictory findings to the above data were reported also.Notably, it has been shown that overexpression of p53 protein is not directly related to HBx protein expression[29]. No correlation was found between the state of HBV DNA and *p53* aberration in China[30] and Europe[31]. In European HCC, it has been reported that underlying, chronic HBV infection does not appear to be associated with an increased rate of p53 overexpression[31]. No significant differences were observed inp53 antigen expression between hepatitis B- and non-hepatitis B- associated hepatocellular carcinomas in Asian patients[32]. The findings of the present study are also in accordance with these observations. This discrepancy among reports for the relationship of p53 overexpression and HBV positivity may be explained by differences of geographical factors, characteristics of cases and methodology used in the studies. To solve this discrepancy, extensive studies including meta-analysis may be required.

In conclusion, although overexpression of p53 was detected in 23.5% (4/17) of HCC, it was not associated with either of 249 mutation or HBV infection. In HCC, overexpression of p53 protein seemed to be related to other than this mutation.

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=국문초록=

간세포암의 *p53* 코돈 249의 돌연변이와 과발현 빈도 및 HBV DNA 양성률

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배경: 간세포암(HCC)에서 *p53* 유전자의 돌연변이의 빈도와 B형 간염 바이러스(HBV)와의 관련성은 지리적 특성이나 위험인자의 유무에 따라 다양하게 나타난다. 본 연구에서는 한국인 HCC에서 *p53* 유전자의 249번 코돈의 변이 및 p53 단백질 과발현 빈도와 B형 간염 바이러스(HBV) DNA 양성률 그리고 이들의 관련성에 대해 알아보고자 하였다.

방법: HCC로 진단된 17개의 검체를 대상으로 p53 발현 정도를 평가하기 위해 단클론성 p53 항체를 이용하여 면역조직화학염색을 실시하였고 *p53* 유전자의 249번 코돈의 돌연변이 유무를 확인하기 위하여 중합효소연쇄반응-제한절편길이다형성을 이용하였으며 HBV를 검출하기 위해 이중중합효소연쇄반응을 실시하였다.

결과: 17개의 HCC 검체에서 코돈 249의 세 번째 염기의 점돌연변이는 관찰되지 않았으나 1개(5.8%)에서 코돈 250 두 번째 염기의 돌연변이를 확인할 수 있었다. p53 면역화학조직염색에서 17개의 HCC검체 중 4개(23.5%)의 검체가 과발현을 보였으며 이들 4 검체 중 3 검체에서 HBV DNA가 양성이었다. HBV DNA는 17개의 검체 중 14개(82.4%)에서 양성을 보였다.

결론: 본 연구의 HCC에서 HBV 감염은 *p53* 249번 코돈의 돌연변이나 p53 과발현과는 관련이 없었다. p53 과발현은 이 돌연변이 외 다른 돌연변이와 관련된 것으로 보인다. **[대한임상미생물학회지 2007;10:84-89]**

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