

Correlation of Mutation and Immunohistochemistry of p53 in Hepatocellular Carcinomas in Korean People

The degree of correlation between sequencing and immunohistochemistry (IHC) for detecting mutations of p53 has not been well established in human hepatocellular carcinoma (HCC). We analyzed 36 HCCs from Korean people for p53 mutation at exons 4-10 by PCR-SSCP and sequencing, and compared the results with the IHC positivity. p53 mutations were identified in 7 out of 36 HCCs (19.4%). These mutations were found widely throughout exons 4-8. No mutation was detected in codon 249. Among the 7 mutations, 6 missense mutations were detected in 15 HCCs with $\geq 5\%$ immunoreactive tumor cells and one nonsense mutation was in 21 HCCs with $< 5\%$ immunoreactive tumor cells. The sensitivity for p53 mutation was 85.7% (6/7), the specificity 69.0% (20/29), the predictive value of positive IHC 40.0% (6/15), and the predictive value of negative IHC 95.2% (20/21). Two missense mutations were detected in 25 cases with $< 10\%$ immunoreactive tumor cells. Predictive values of both positive IHC and negative IHC were higher in $\geq 5\%$ overexpression group than in $\geq 10\%$ overexpression group or $> 0\%$ overexpression group. This study suggests that 5% immunoreactivity is a reliable immunohistochemical threshold value to detect p53 mutations in HCCs and the spectrum of p53 mutations in HCCs in Korean people is different from that of high aflatoxin B₁ exposure areas.

Key Words : Carcinoma, Hepatocellular; Genes, p53; Immunohistochemistry; Mutation

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INTRODUCTION

The p53 tumor suppressor gene has a critical role for regulation of cell cycle, cellular growth, and maintenance of genomic integrity (1). And it is well known that the inactivating mutation of p53 is the most common genetic alteration in human cancers including hepatocellular carcinoma (HCC) (2). The mutational spectrum of p53 has been reported to differ in HCCs from different geographic regions. For example, the G to T transversion at the third position of codon 249 has been detected very frequently, ranging from 30 to 58% of HCCs, in southern Africa and southern China in which aflatoxin B₁ is contaminated highly in food and hepatitis B virus (HBV) infection is endemic (3-5). In contrast, few or none of the mutations occur at codon 249 in low aflatoxin B₁ exposure areas (6-9). In Korea, HCC is one of the most common cancers, HBV-related HCC constitutes about 65-70% of HCC, and dietary exposure of aflatoxin B₁ is low (10, 11). There are a few reports, however, on the status of p53 mutation at exons 3-9 or 5-8 in HCC patients in Korea (12-14).

The missense mutations of p53 may result in biologically altered proteins with increased stability that is easily detectable by immunohistochemical method. However, deletion or trun-

cated proteins resulted from nonsense or frameshift mutations are usually not detectable by immunohistochemical method, because these proteins are very unstable in the cell, even using antibody containing the corresponding N-terminal epitope (2). Therefore, immunohistochemistry (IHC) will be less sensitive in detecting mutations in tumors with high proportion of these non-missense mutations. p53 overexpression is detected at a higher incidence than gene mutation in human cancers (44% vs 36%) (2).

In p53 IHC, the proportion of cells that show immunoreactivity is very important (15). Occurrence of just occasional strongly immunoreactive cells in a tumor does not seem to correlate with obvious molecular abnormality of p53 (16). They may rather represent the normal working of the p53 system in which the wild-type protein accumulate in response to spontaneous genetic errors occurring at a higher frequency in the tumor than in the normal surrounding tissue. In p53 immunohistochemical study on HCC, the threshold value above which the result is regarded positive has not been well established. p53 overexpression in HCC is not always dependent on p53 mutation (17). And the degree of correlation between sequencing and IHC for detecting mutations of p53 has not been well established in human HCCs.

In this study, we determined what proportion of cells that show immunoreactivity is a reliable immunohistochemical threshold value to detect *p53* mutations in HCCs and evaluated the spectrum of *p53* mutations at exons 4-10 in 36 HCCs from Korean people.

MATERIALS AND METHODS

Tumor samples

Samples of 36 HCCs were obtained from 34 Korean patients at the time of curative surgical resection at Samsung Medical Center, Seoul, Korea, between 1999 and 2000. None of the patients had any preoperative chemotherapy. All of these were primary HCC tumors: 33 were from patients with a single HCC nodule and 3 were from 1 patient with three HCC nodules of Edmondson & Steiner grade I. Informed consent was obtained from each patient. The age of patients ranged from 26 to 89 yr, with a mean age of 51.8 yr. The male-to-female ratio was 27:7. All samples included both tumors and nontumorous tissues. Halves of the samples were snap-frozen in liquid nitrogen and stored at -80°C until DNA extraction. The other halves were fixed in 10% formalin and embedded in paraffin. The longest diameter of a tumor ranged from 9 to 110 mm, with a mean of 38.6 mm. The degrees of differentiation of tumor cells were determined according to the Edmondson & Steiner's grading system (18). There were 6 cases of Edmondson & Steiner grade I, 11 grade II, and 19 grade III. The nontumorous liver showed cirrhosis in 23 (67.6%) patients and chronic hepatitis in 11 (32.4%). The etiologies of liver disease were HBV in 27 patients, hepatitis C virus (HCV) in 3, and cryptogenic in 4.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections of 4 μm containing both HCC and nontumorous liver were prepared. Immunohistochemical study was performed using the streptavidin-biotin complex method and TechMate™ 1,000 automated staining system (DakoChemmate, Glostrup, Denmark). Primary monoclonal antibody against *p53* (clone BP53-12) was purchased from Zymed Lab Inc. (San Francisco, CA, U.S.A.) and used at 1:80 dilution. Deparaffinized sections were processed in 0.05 M sodium citrate buffer (pH 6.0) and heated in a microwave oven for 10 min for antigen retrieval. Sections were then incubated with the primary antibody for 60 min at room temperature. DAB (3,3'-diaminobenzidine tetrahydrochloride) was used as chromogen. Negative controls were run simultaneously with an omission of primary antibody. Blocks of normal liver were prepared from 10 patients with metastatic colonic carcinoma of the liver as control cases.

For assessment of the positivity of immunostaining for each section, only nuclear staining was regarded as positive. We

counted tumor cells with clearly brown reaction products in nuclei by monitoring at least 1,000 tumor cells from more than five high power fields where positive cells were present at a relatively uniform density. Three observers evaluated staining results independently and differences in interpretation were resolved by consensus.

DNA extraction

Frozen tissue samples were pulverized to a powder using a mortar and pestle precooled with liquid nitrogen, suspended in lysis buffer, and treated with proteinase K. DNA was extracted by phenol-chloroform-isoamyl alcohol as described elsewhere (19).

PCR-SSCP and DNA sequencing

Each of *p53* exons 4-10 was amplified by PCR. The primers and the cycling conditions for each exon are listed in Table 1. Each PCR reaction was generally performed under standard conditions in a 10 μL reaction mixture containing 1 μL of template DNA, 0.4 μM of each primer, 125 μM of each dNTP, 1.5 mM MgCl_2 , 0.4 unit of *Taq* polymerase, 0.5 μCi of [^{32}P] dCTP (Amersham, Buckinghamshire, U.K.), and 1 μL of $10 \times$ buffer. The reaction mixture was predenatured for 12 min at 95°C and amplified for 35 cycles (denaturing for 30 sec at 95°C , annealing for 30 sec at $55\text{--}59^{\circ}\text{C}$, and extending for 30 sec at 72°C). Final extension was continued for 5 min at 72°C . PCR products were denatured for 5 min at 95°C at a 1:1 dilution of formamide loading dye containing 95% formamide, 20 mM/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol and were loaded onto a MDE gel (AT Biochem, Malvern, PA, U.S.A.) with 10% glycerol. After electrophoresis, the gels were transferred to 3-mm Whatman paper and autoradiography was performed using Kodak X-OMAT film (Eastman Kodak, Rochester, NY, U.S.A.). For the detection of

Table 1. *p53* primer sequences and PCR cycling conditions

Exon	Primers*	Product size	Annealing Temp.
4	(1)ATCTACAGTCCCCCTTGCCG (2)GCAACTGACCGTGCAAGTCA	296	55°C
5	(1)GCTGCCGTGTTCCAGTTGCT (2)CCAGCCCTGTCGTCTCTCCA	294	58°C
6	(1)GGCCTCTGATTCCTCACTGA (2)GCCACTGACAACCACCCTTA	199	55°C
7	(1)TGCCACAGGTCTCCCCAAGG (2)AGTGTGCAGGGTGCAAGTG	196	56°C
8	(1)CCTTACTGCCTCTTGCTTCT (2)ATAACTGCACCCTTGGTCTC	225	55°C
9	(1)GCCTCAGATTCACITTTATCACC (2)CTTTCCACTTGATAAGAGGTCCC	152	56°C
10	(1)ATACTTACTTCTCCCCCTCTC (2)AGGAAGGCAGGGGAGTAG	184	59°C

*(1), sense primer; (2), antisense primer.

mutations, DNAs showing mobility shifts were cut out from the dried gels and reamplified for 30 cycles using the same primer set. Sequencing of the PCR products was carried out using the cyclic sequencing kit (Perkin-Elmer, Foster City, CA, U.S.A.) according to the manufacturer's recommendations. All mutations were verified through triplicated experiments including PCR-SSCP and sequencing analysis, which ensured the specificity of the results.

Statistical analysis

Chi-square test was used for the statistical analysis. Two-sided *p*-value of less than 0.05 was considered as statistically significant.

The sensitivity of *p53* mutation was the percentage of IHC-positive tumors among tumors with identified mutation. The specificity was the percentage of IHC-negative tumors among tumors without *p53* mutation. The predictive value of positive IHC was the percentage of IHC-positive tumors, which had *p53* mutation. The predictive value of negative IHC was the percentage of IHC-negative tumors, which lacked *p53* mutation. Accuracy was the percentage of tumors correctly classified for *p53* mutation by IHC.

RESULTS

Immunohistochemical analysis of p53

Of the 36 HCC cases, 3 had ≥50% of tumor cells stained for p53, 8 had 10-49% of tumor cells stained for p53, 4 had 5-9% of tumor cells stained for p53, 10 had 1-4% of tumor cells stained for p53, and 11 did not stained for p53. No nuclear staining was found in nontumorous liver tissues or normal controls.

Fifteen out of 36 HCCs (41.7%) had ≥5% of tumor cells stained for p53: 13 out of 27 HBsAg seropositive cases (48.1%), 1 out of 5 anti-HCV antibody seropositive cases (20%), and 1 out of 4 both HBsAg and anti-HCV antibody seronegative cases (25%). The p53 was overexpressed more frequently in HCCs with poorer histological grade of differentiation (Ed-

mondson I, 0%; Edmondson II, 36.3%; Edmondson III, 57.9%; *p*=0.039).

DNA sequencing analysis

We detected 7 somatic mutations out of 36 HCCs (19.4%) consisting of 6 missense and 1 nonsense mutations (Table 2). These mutations were found widely throughout exons 4-8 without any mutational hot spot. No mutation was detected in codon 249. Two representative cases with aberrant bands and mutations are shown in Fig. 1. Nontumorous liver tissues from all 36 cases showed no mutation.

Mutations were found in 5 HBsAg seropositive cases, 1 anti-HCV antibody seropositive case, and 1 both HBsAg and anti-HCV antibody seronegative case. Mutations were frequently found in HCCs with poorer histological grade of differentiation (Edmondson I, 0%; Edmondson II, 18.2%; Edmondson III 26.3%; *p*>0.05).

Correlation of p53 mutation and p53 overexpression

Among the 7 mutations, 6 missense mutations were detected in 15 HCCs with ≥5% immunoreactive tumor cells and one nonsense mutation was in 21 HCCs with <5% immunoreactive tumor cells (40% vs 4.8%, *p*=0.027). The sensitivity for *p53* mutation was 85.7% (6/7), the specificity 69.0% (20/29), the predictive value of positive IHC 40.0% (6/15), the predictive value of negative IHC 95.2% (20/21), and the accuracy 72.2% (26/36).

No missense mutation was detected in all 21 cases with <5% immunoreactive tumor cells but 2 missense mutations were detected in 25 cases with <10% immunoreactive tumor cells.

Table 2. Summary of *p53* mutations in 36 cases of hepatocellular carcinoma

Case No.	Exon	Codon	Nucleotide change	Amino acid change	Type of mutation	Hepatitis* virus
10	4	111	CTG→CGG	Leu→Arg	Transversion	HCV
11	6	194	CTT→CGT	Leu→Arg	Transversion	-
12	5	175	CGC→CTC	Arg→Leu	Transversion	HBV
22	5	158	CGC→CAC	Arg→His	Transition	HBV
30	8	280	AGA→TGA	Arg→Stop	Transversion	HBV
33	8	273	CGT→TGT	Arg→Cys	Transition	HBV
35	6	214	CAT→CGT	His→Arg	Transition	HBV

*HBV, HBsAg(+); HCV, anti-HCV antibody(+).

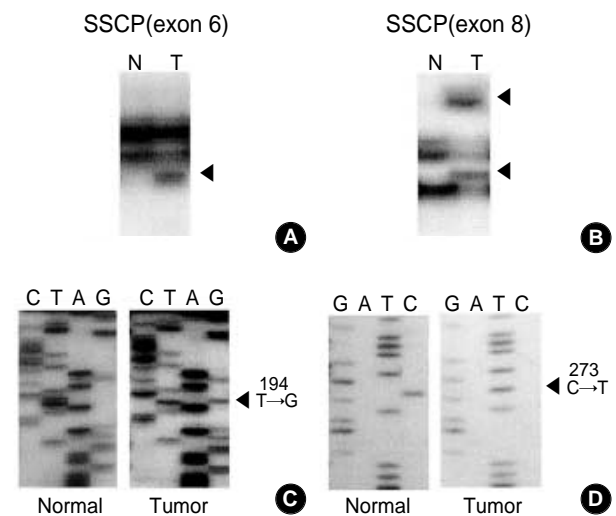


Fig. 1. PCR-SSCP of exon 6 and 8 of the *p53* shows abnormally shifted bands (arrowheads) in A (case 11) and B (case 33) (N, normal; T, tumor). Sequencing reveals CTT→CGT at codon 194 in case 11 (C) and CGT→TGT at codon 273 in case 33 (D).

If we regarded the p53 IHC as positive when $\geq 10\%$ of tumor cells were stained, the sensitivity for p53 mutation was 57.1% (4/7), the specificity 75.9% (22/29), the predictive value of positive IHC 36.4% (4/11), the predictive value of negative IHC 88.0% (22/25), and the accuracy 72.2% (26/36). If we regarded the p53 IHC as positive based on the presence of any immunoreactive tumor cells, the sensitivity for p53 mutation was 85.7% (6/7), the specificity 34.5% (10/29), the predictive value of positive IHC 24.0% (6/25), the predictive value of negative IHC 90.9% (10/11), and the accuracy 44.4% (16/36).

DISCUSSION

The p53 consists of 11 exons. Greenblatt et al. (2) had identified 50 studies in which sequencing of the entire coding region of p53 in human cancers was reported. Of the 560 mutations reported in these papers, 87% were in exons 5-8 and most of the others were in exon 4 (8%) and exon 10 (4%). Mutations outside exons 5-8 were most frequent in urinary bladder carcinoma (28%) and HCC (24%). There have been six studies in which sequencing of the entire coding region of p53 in HCCs was reported (6, 17, 20-23). All of the 68 mutations reported in these papers were in exons 4-10. Therefore, we analyzed nucleotide sequences of the p53 in exons 4-10.

The frequency of p53 mutations has been reported to differ in HCCs from various geographic areas: 50-58% in southern China and southern Africa, 15-32% in Japan, and 15.4% in Germany (3, 5, 6, 8, 24). In this study, we showed that p53 mutation in HCCs in Korean people, at a frequency of 19.4%, is not uncommon. The not uncommon frequency of p53 mutations might represent only one of the multiple steps involved in hepatocarcinogenesis, as in other human cancers (1). In addition, no mutation was detected at codon 249 in the present study. This is consistent with the low prevalence of mutations at codon 249 reported in geographic areas where aflatoxin B₁ is not detectable at high levels in the diet, such as U.S.A., Germany, France, Britain, Taiwan, Australia, Japan, and Korea (6-9, 12-14, 25-27). This finding suggests that factors other than aflatoxin B₁ may be responsible for the p53 abnormalities in HCCs. The mutations in HCCs in low aflatoxin exposure areas often show a wide distribution over the highly conserved region covering exons 4-10 (6). The findings of the present study are also in line with this observation, with mutations scattered over exons 4-8.

We have found that overexpression of p53 in HCCs was correlated with the mutation of the p53 ($p=0.027$). However, there was substantial discrepancy between molecular genetic alterations in p53 and overexpression of the protein. In p53 immunohistochemical studies on HCCs, there have been different threshold values above which the result is regarded positive: presence of any labeled cells, 2%, 5%, 10%, or 50% immunoreactivity (28-32). Volkmann et al. (33) regarded the

p53 IHC as positive based on the presence of any immunoreactive cells and reported that one HCC with a few immunoreactive cells had wild-type p53. Volkmann et al. (34) regarded the p53 IHC as positive based on the presence of any immunoreactive cells and reported that one out of eight HCCs with 1-9% immunoreactive cells showed missense mutation of p53. Hsia et al. (35) regarded 10% immunoreactivity as the threshold value and reported that one out of four HCCs with 1-9% immunoreactive cells showed missense mutation of p53.

Our findings showed that HCCs with $\geq 5\%$ immunoreactive tumor cells are the ones in which p53 mutations are most frequently present. No missense mutation was detected in all 21 cases with $<5\%$ immunoreactive tumor cells but 2 missense mutations were detected in 25 cases with $<10\%$ immunoreactive tumor cells. Predictive values of both positive IHC and negative IHC were higher in $\geq 5\%$ overexpression group than in $\geq 10\%$ overexpression group or $>0\%$ overexpression group. The specificity for p53 mutation in $>0\%$ overexpression group was very low. These findings suggest that 5% immunoreactivity is a reliable immunohistochemical threshold value to detect p53 mutations in HCCs. It should be noted that our findings might not be applicable to other tumor types in which discordance between IHC and molecular genetic analysis of p53 is more frequent.

These results suggest that 5% immunoreactivity is a reliable immunohistochemical threshold value to detect p53 mutations in HCCs and the spectrum of p53 mutations in HCCs in Korean people is different from that of high aflatoxin B₁ exposure areas.

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