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ORIGINAL ARTICLE

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p53 Mutation and overexpression in hepatocellular carcinoma and dysplastic nodules in the liver

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Abstract In hepatocarcinogenesis, both de novo and multistep pathways have been suggested and in the latter a dysplastic nodule is the proposed precancerous lesion. In this study, we tried to ascertain whether or not the *p53* gene is altered in low-grade/high-grade dysplastic nodules (LDN/HDN) and to determine the role of *p53* alteration in multistep hepatocarcinogenesis. Eight hepatocellular carcinomas (HCCs), 9 HDNs, 17 LDNs and 25 cirrhotic nodules (LCs) were examined by polymerase chain reaction-single strand conformation polymorphism/direct sequencing and immunohistochemical staining for *p53*. Four of the 8 HCCs (50%) revealed *p53* overexpression and 2 (25%) had missense mutations. Four of the 9 HDNs (44%) showed weak and/or focal *p53* overexpression but none had mutation in the exons examined. Neither *p53* overexpression nor mutation was found in 17 LDNs and 25 LCs. These results suggest that *p53* mutation might be an unusual event in precancerous lesions of multistep hepatocarcinogenesis (DN-HCC sequence) and may play a less crucial part than in colorectal carcinogenesis.

Key words *p53* Mutation · Overexpression · Dysplastic nodule · Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent neoplasms in East Asia, including Korea [1]. Hepatitis B and C viral infection, aflatoxin exposure and cirrhosis are known risk factors [2, 3], but the precise cellular and molecular mechanisms of the development of HCC are still unclear. Dysplastic nodules (DN; adenomatous hyperplasia or macroregenerative nodule) in cirrhotic liver have recently been described as possible precancerous lesions in multistep hepatocarcinogenesis [4–9].

The molecular aspects of sequential genetic alterations have been reported in multistep carcinogenesis in many organs, including the colon, stomach, oesophagus and lung [10–15]. One such alteration is mutation of the *p53* tumour suppressor gene, now known to be the most commonly recognized genetic abnormality in human cancers and in precancerous lesions [16]. In HCC, different geographical regions show varying *p53* mutation rates: over 50% in China and South Africa, 20–30% in Japan and under 10% in the U.S. [17]. Our previous study revealed *p53* mutation in 33% of HCCs (data not shown). Two recent reports have shown an increased frequency of mutation of *p53* codon 249 in nontumorous livers from Qidong, China [18] and *p53* expression in cirrhotic nodules [19]. There has been only one publication on mutant p53 protein expression in adenomatous hyperplasia of the liver [20], however. The concept of a premalignant nature of the DN originated from the observation that in DNs, the genesis of some HCCs involved the nodule-in-nodule pattern [4, 6]. DNs were further divided into low-grade and high-grade DN (LDN and HDN). Several histological criteria are used in this discrimination, the most important being that LDN revealed minimal cytological atypia and mildly increased cellularity, forming clonelike populations, whereas HDN showed a high nuclear-to-cytoplasmic ratio, nuclear hyperchromasia, and/or a slightly irregular trabecular arrangement; that is cytological and structural atypia [9]. From the pathological point of view, the presence of atypia suggests an increased risk of malignant transformation and our question is “Is there any causative genetic alteration prior to or concurrent with this atypia?” In this study, we tried to resolve this question by assaying *p53* alteration in HCCs, HDNs, LDNs and LCs.

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Materials and methods

A total of 26 DNs (17 LDNs and 9 HDNs) were derived from 20 surgically resected livers at Seoul National University and Inje University Seoul Paik Hospital between 1990 and 1995. They were all reviewed by three pathologists (Y. K. K., G. H. K., and Y. I. K.). DNs were classified according to the International Working Party's criteria into two groups: LDN and HDN [9]. LDNs were composed of minimally atypical hepatocytes with slightly increased cellularity, whereas HDNs showed cellular atypia, with an irregular trabecular and/or pseudoglandular arrangement. Large regenerative nodules (LRNs) were not included in the DN group, but were considered to be large cirrhotic nodules (≥0.8 cm in diameter). In 12 cases, DNs were separate lesions in the background liver adjacent to HCCs, and 7 of these HCCs were available for study. A case of HDN had central well-differentiated hepatocellular carcinoma foci (W-D HCC), and each HDN and W-D HCC was examined. In addition, 25 cirrhotic nodules (LCs), including 5 large regenerative nodules, were analysed. Archival specimens comprising formalin-fixed paraffin-embedded tissue blocks were obtained from each lesion.

Genomic DNA was isolated from five or six, 10-µm-thick unstained sections trimmed from each lesion. Haematoxylin-eosin-stained sections were reviewed concomitantly to facilitate accurate identification of the lesions. After deparaffinization by three washes in xylene and concentrated alcohol, DNA extraction was performed using a kit (G nome DNA isolation kit, BIO101).

Amplification of DNA (exons 5–8 of *p53*) using the polymerase chain reaction was performed. Because formalin fixation induces single-strand breakage of DNA [21], the oligonucleotide primers, having sequences corresponding to those mainly within the exons, were designed on the basis of published sequences [11]. The primers used were as follows: exon 5, 5'-TACTCCCCTGCCCTCAACAA-3' and 5'-CATCGCTATCTGAGCAGCGC-3'; exon 6, 5'-GTCTGGCCCCTCCTCAGCAT-3' and 5'-CTCAGGCGGCTCATAGGGCA-3'; exon 7, 5'-TCTGACTGTACCACCATCCA-3' and 5'-CTGGAGTCTTCCAGTGTGAT-3'; exon 8, 5'-TGGTAATCTACTGGGACGGA-3' and 5'-GTCCTGCTTGCTTACCTCGC-3'. Prior to SSCP analysis, products of each PCR containing all components except [α-^{32}P]dCTP were confirmed by agarose gel electrophoresis. The 10-µl reaction volume contained up to 50 ng genomic DNA, 10×Taq polymerase buffer (Promega, Madison), 0.4 pmol of each primer, 0.2 mM of each deoxynucleotide triphosphate, and 0.5 U Taq polymerase (Promega). SSCP was performed directly by adding 1.5 µCi of [α-^{32}P]dCTP to the PCR reaction. Using a thermal cycler (Perkin Elmer Cetus, version 2.0), the reaction mixtures underwent PCR: initial denaturation (94°C, 3 min), 34 amplification cycles of denaturation (95°C, 30 s) annealing (55–60°C, 30 s) and extension (72°C, 30 s), followed by 72°C conditions for 10 min. One-fifth volume of 100 mM EDTA/1.0% sodium dodecyl sulfate was added to each completed reaction, and then 1 µl of the solution was mixed with 1 µl of dye mix (95% formamide–20 mM EDTA–0.05% bromophenol blue–0.05% xylene cyanol), denatured at 90°C for 4 min, and applied (2 µl/lane) to nondenaturing 8% or 6% polyacrylamide gel with or without 5% glycerol. Electrophoresis was performed at 30 W for 2–3 h at 4°C, using a sequencing-type apparatus (Kodak Biomax STS-45i Sequencer). The gel was dried on 3 M filter paper (Whatman) for 30 min and exposed to X-ray film (Kodak XRP-1) at –78°C overnight. Positive controls for each exon were 1421T and 5564T (from resected stomach cancer tissue) for exons 5 and 6, and LS1034 and Widr (cell lines) for exons 7 and 8, respectively. The negative control was fetal liver tissue.

For immunohistochemical (IHC) staining, 4-µm-thick sections from each lesion were stained with mouse monoclonal antibody (DO-7, Dako). The sections were deparaffinized in xylene and hydrated in alcohol gradient. For antigenic retrieval they were immersed in citric acid buffer (pH 6.0) and boiled in a microwave oven for 5 min. After washing, the sections were treated with rabbit serum, and DO-7 monoclonal antibody was added at 1:100 dilution. The sections were incubated for 12 h at 4°C and then incubated with biotinylated anti-mouse immunoglobulin and avidin–biotin-peroxidase complex (both from Dako). The colour reaction for peroxidase was developed in acetyl ethyl carbazole and 0.03% hydrogen peroxide. Fetal liver and colonic carcinoma sections were used for external negative and positive controls. Results were recorded according to the percentage of cells showing positive staining (–,<20%, 20%<&<50%, and >50%) and intensity of nuclear staining (–, ±, and +).

Sequence analysis was performed on samples with abnormal bands detected by SSCP analysis. The purified PCR product (from 80 µl reaction volume) in each exon was sequenced using Sanger's dideoxy method, using a sequencing kit (Sequitherm, Epicentri Technology), [α-^{35}S] labelled dATP and the same sense or antisense primer as had been used for PCR amplification.

Results

Of the 20 cases in this series, 16 were men and 4 were women. The age distribution was from 47 to 67 years (mean 56 years). There were 17 with positive serum HBsAg, 2 with positive HBcAb and 2 with anti-HCV.

DNs presented separately in the vicinity of HCCs (12/13) or coexisted with the central grade I HCC component (1/13). None of the 7 (of 12) earlier HCCs available for analysis showed evidence of coexisting DN. The size of the 13 HCCs was 1.4–6.0 cm (mean 3.2 cm), and the 8 HCCs examined measured 2.8–4.5 cm (mean 3.4 cm). In 6 patients DNs were multiple; in 1, there were two HDNs, 2 patients had HDN and LDN, and 3 had two LDNs. The sizes of 9 HDNs were 0.5–2.8 cm (mean 1.48 cm) and 17 LDNs were 0.8–1.8 cm (mean 1.08 cm). There were diffuse fatty changes in 3 HDNs, and 1 had a focal pseudoacinar arrangement. Five large regenerative nodules (more than 0.8 cm in diameter) with the same microscopic features as the surrounding cirrhotic nodules were noted in 3 cases.

The results of DNA sequencing and immunohistochemical studies are summarized in Tables 1 and 2.

Electrophoretic mobility shifts on PCR-SSCP analysis were noted in 2 (25%) of the 8 HCCs (1 in exon 5 and 1 in exon 8; Fig. 1A, B). Sequencing confirmed two missense mutations (Fig. 1C, D): one had a G-to-T transversion at codon 154 (GGC to GTC) and the other had a T-to-G transversion at codon 274 (GTT to GGT). No definite mobility shift was detected in 9 HDNs, 17 LDNs or 25 LCs. Abnormal nuclear expression of *p53* was detected in 4 (50%) of the 8 HCCs: 3 demonstrated >50%, + (2 of them had *p53* mutation) and 1 20<&<50%, ± staining (Fig. 2). Of the nine HDNs, 4 revealed weak *p53* overex-

Table 1 *p53* Mutation and overexpression in cases of cirrhotic nodules (*LC*), low-grade and high-grade dysplastic nodules (*LDN* and *HDN*) and (well-differentiated *W-D*) hepatocellular carcinoma (*HCC*)

	p53 Mutation	*p53* Overexpression
LC	0/25 (0%)	0/25 (0%)
LDN	0/17 (0%)	0/17 (0%)
HDN	0/9 (0%)	4/9 (44%)
HCC	2/8 (25%)	4/8 (50%)
W-D HCC	0/1	0/1

Table 2 Summary of profiles of HCCs and dysplastic nodules in individual cases (*NE* not evaluated)

Case	Diagnosis	Size (cm)	*p53* Mutation	*p53* Overexpression[b]
1	HCC	1.4	NE	NE
	HDN	1.2	–	–
2[a]	W-D HCC	2.8	–	–
	HDN	2.8	–	–
3	HCC	3.5	274 (GTT → GGT): Val → Gly	>50%, +
	LDN	1.0	–	–
	LDN	0.9	–	–
4	HDN	1.5	–	–
5	HCC	4.5	–	>50%, +
	HDN	1.5	–	20<&<50%, ±
	LDN	0.9	–	–
6	LDN	1.5	–	–
7	HCC	3.0	–	–
	HDN	1.0	–	–
8	HCC	3.5	NE	NE
	LDN	0.9	–	–
9	LDN	1.5	–	–
10	LDN	0.8	–	–
11	LDN	1.0	–	–
12	HCC	6.0	NE	NE
	LDN	1.0	–	–
13	LDN	1.5	–	–
14	LDN	0.9	–	–
15	HCC	2.0	NE	NE
	HDN	2.2	–	<20%, ±
16	HCC	1.8	NE	NE
	LDN	0.9	–	–
	LDN	0.8	–	–
17	HCC	3.5	–	–
	LDN	1.8	–	–
	LDN	0.8	–	–
18	HCC	3.0	–	20<&<50%, ±
	HDN	1.7	–	–
	LDN	1.2	–	–
19	HCC	3.3	154 (GGC → GTC): Gly → Val	>50%, +
	HDN	0.9	–	>50%, ±
	HDN	0.5	–	20<&<50%, ±
20	HCC	3.6	–	–
	LDN	1.0	–	–

[a] In case 2, W-D HCC and surrounding HDN were examined simultaneously
[b] The results for *p53* overexpression were recorded according to the percentage of positive cells (–,<20%, 20%<&<50%, and >50%) and intensity of nuclear staining (–, ±, and +)

pression: 1 showed >50%, ±, and 2 20<&<50%, ± staining; there was localized staining (<20%, ±) of *p53* in the remaining HDN (Fig. 2). Among the 4 HDNs with *p53* overexpression, 2 were multiple nodules from a patient whose HCC had *p53* mutation and overexpression (case 19); 1 had a neighbouring HCC with only *p53* overexpression (case 5); in the remaining 1 (case 15) the accompanying HCC was not available. No *p53* overexpression was noted in 17 LDNs and 25 LCs.

Discussion

In hepatocarcinogenesis, both de novo and multistep pathways have been suggested, and in the latter the dysplastic nodule is the proposed precancerous lesion [4–9]. Previously, terms such as adenomatous hyperplasia (ordinary and atypical) or macroregenerative nodule (type I and type II) were used [4–8], but we use the term dysplastic nodule, low-grade and high-grade (LDN and HDN), following the International Working Party's criteria. These defined the lesion as a nodular region of hepatocytes at least 1 mm in diameter, with dysplasia but without definite histological criteria of malignancy [9]. Sequential development of LDN, HDN and early HCC in a multistep hepatocarcinogenesis model is very similar to multistep tumorigenesis in other organs, such as the colon [10]. Several previous studies have identified morphological characteristics, proliferative activity and DNA-ploidy of DN [4–8]. Recently, monoclonality of DN was confirmed by restriction fragment length polymorphism of the X-linked gene, DNA fingerprinting and loss of heterozygosity study [22].

p53 gene mutation is one of the best known and most common cancer-related genetic changes in many human tumours [16]. Mutation of this gene removes the regulatory function of wild-type *p53* in cellular proliferation and results in a growth advantage and in neoplastic transformation [16]. Mutation and/or overexpression of *p53* has been reported in precancerous lesions such as Barrett's epithelium, and colonic and gastric adenomas [10–14]. In colorectal carcinogenesis, *p53* mutation occurs in about 20% of adenomas and seems to be a rate-limiting step that makes an important contribution to tumour progression [23]. Although the *p53* mutation rate in HCC is not as high as in colonic carcinoma, several recent reports

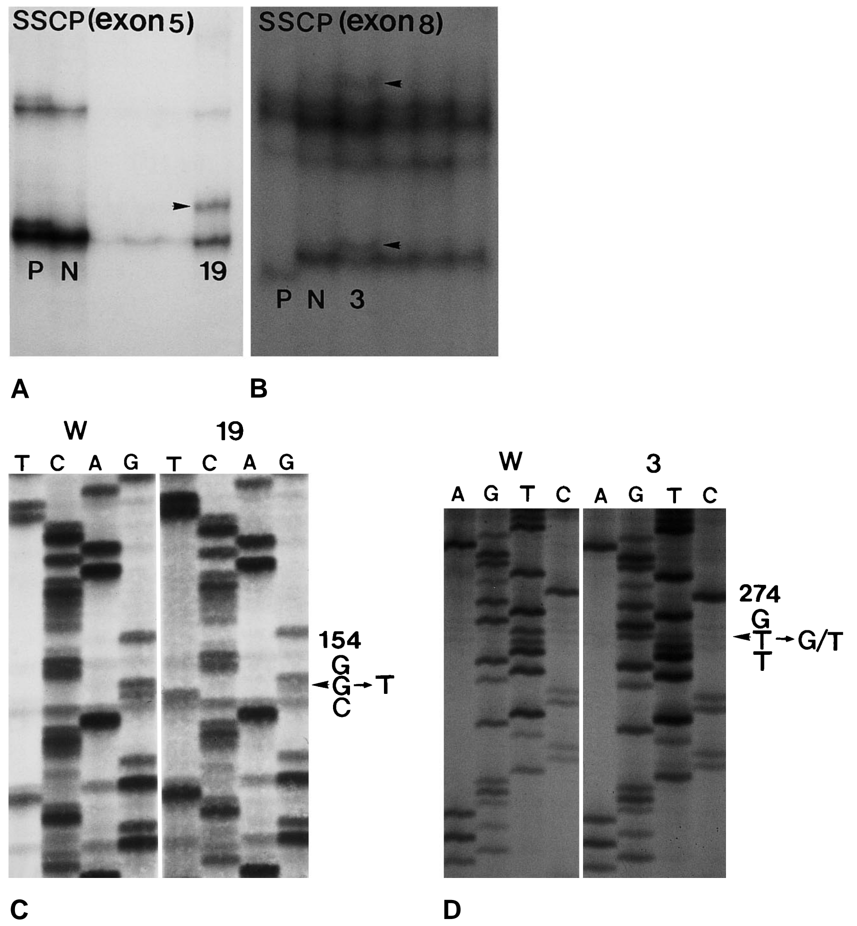

Fig. 1 PCR-SSCP of exons 5 and 8 of the *p53* gene show abnormally shifted bands *(arrowheads)* in **A** case 19 and **B** case 3 *(P* positive control, *N* negative control). Direct sequencing reveals **C** GGC → GTC at codon 154 in case 19 and **D** GTT → GGT at codon 274 in case 3 *(W* wild type)

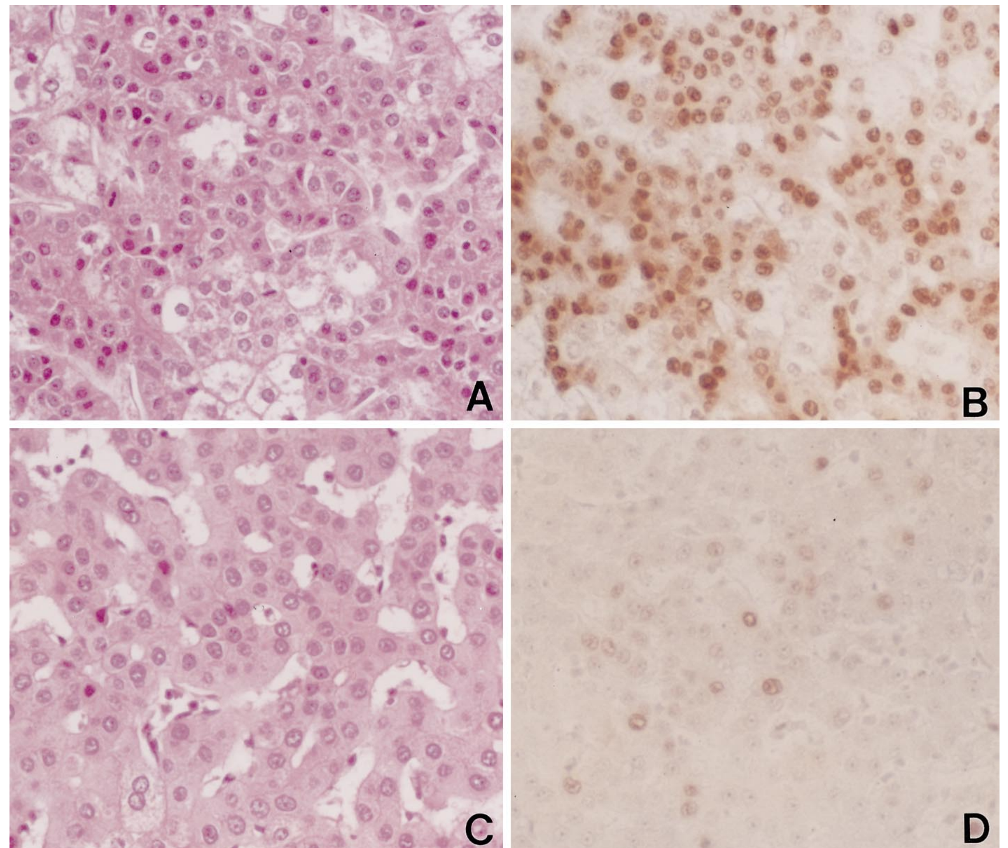

Fig. 2 *Upper panels* reveal **A** hepatocellular carcinoma, **B** with diffuse positive staining for *p53*. **C** A case of high-grade dysplastic nodule, **D** showing focal *p53* overexpression is presented in the *lower panels*. **A, C** HE, ×200; **B, D** ABC, ×200

have suggested that stepwise accumulation of *p53* mutation may occur before the development of HCC [18–20].

This study sought to ascertain whether or not the *p53* gene is altered in DN and to determine the role and the time of *p53* alteration in multistep hepatocarcinogenesis. We used 26 DNs (17 LDNs and 9 HDNs), 8 HCCs including 1 W-D HCC, and 25 LCs from 20 patients. p53 Mutation was detected in 2/8 (25%) of HCCs, but not in any of the HDNs, LDNs and LCs. The information derived from our mutation assay has some limitations. There may have been certain mutations that we missed (splicing, promoter, mutations in other exons or outside the primers), because only exonal sequences from exons 5–8 were analysed. However, mutations outside exons 5–8 have been noted less frequently than mutations inside (the comparative prevalence of mutation inside to outside exons 5–8 in HCC was estimated as about 76–24 [16]). We therefore suggest that unlike carcinogenesis in other organs, such as the colon, *p53* mutation might be a very unusual event in the precancerous lesions of multistep hepatocarcinogenesis and may have a less crucial role in the DN-HCC sequence.

p53 Overexpression was noted in 4 of the 8 (50%) HCCs, with a diffuse strong pattern in 3 instances and weak and focal staining in the other. Weak and/or focal *p53* overexpression was noted in 4 of the 9 HDNs; no *p53* overexpression was found in LDNs and LCs. There may thus be certain mechanisms that stabilize *p53* in the HDN stage. The results have some similarity with those of a previous study, which described a significant correlation between *p53* overexpression and the degree of dysplasia in colorectal adenoma [14]. With regard to p53 overexpression, our results differ from those of Livini et al. [19] and Zou et al. [20] concerning *p53* expression in cirrhotic nodules, probably because of the geographical difference and the different antibody used.

Overexpression of *p53* on IHC staining, but without an abnormal shifted band in SSCP, was noted in 2 HCCs and 4 HDNs; several explanations may account for these SSCP(–)/IHC(+ or ±) cases. Some mutations may have occurred outside exons 5–8, and the SSCP technique may not be 100% sensitive [16, 24]. However, accumulation of wild-type p53 protein in the absence of gene mutation may also be expected, because DO-7 reacts with both wild and mutant p53 protein. The overexpression of wild-type *p53* may be either pathological (nonfunctional, by forming complexes with other molecules or mutation of *p53* downstream effector gene, such as *WAF-1*, with loss of feedback control) or physiological (functional *p53* activated in DNA-damaging conditions) [16, 19, 24]. There is need for a precise molecular study to investigate the above possibilities, especially for the IHC(±) HDNs.

The timing of *p53* mutation in multistep hepatocarcinogenesis could not be thoroughly evaluated because only 1 HCC coexisted with background HDN, and both were SSCP(–)/IHC(–). Since SSCP(+)/IHC(+ or ±) HCCs did not show direct evidence of a nodule-in-nodule pattern, we were unable to determine how they had been formed. Extended study is needed to determine the precise point in multistep hepatocarcinogenesis at which *p53* mutation occurs.

In summary, *p53* mutation may be an unusual event in the precancerous lesions of multistep hepatocarcinogenesis and may have a less crucial role in the DN-HCC sequence than in colorectal adenoma-carcinoma. We suggest there may be some epigenetic phenomena that stabilize *p53* in HDN nodules.

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