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Mutation Pattern of the *p53* Gene as a Diagnostic Marker for Multiple Hepatocellular Carcinoma[1]

Tatsuya Oda,[2] Hitoshi Tsuda, Aldo Scarpa,[3] Michiie Sakamoto, and Setsuo Hirohashi[4]

Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104 [T. O., H. T., A. S., M. S., S. H.], and Clinical Medicine, Surgery, Tsukuba University, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305 [T. O.], Japan

ABSTRACT

Hepatocellular carcinoma, sometimes shows multiple tumor nodules, therefore poses a problem of differential diagnosis between cancers of multifocal and those of metastatic origin. Conventionally, pathological criteria have been used for this purpose, but these are largely subjective. In order to facilitate more objective differential diagnosis of multiple hepatocellular carcinoma, we used the pattern of mutation of the *p53* gene as a marker for each tumor nodule. We studied 58 nodules from 26 cases of multiple hepatocellular carcinoma using polymerase chain reaction-single strand conformation polymorphism analysis, a simple method for detecting mutations. *p53* gene mutations were detected in 65% (17 of 26) of cases. The internodule mutation patterns were heterogeneous in 11 cases and homogeneous in 6, enabling a multifocal origin to be diagnosed in the former and a metastatic origin in the latter at the genetic level. Moreover, the origin of recurrent tumors was determined from the mutation pattern. It is concluded that analysis of *p53* mutations seems to be useful for differentiating the origin of multiple cancers, since the information it yields is essentially objective.

INTRODUCTION

The frequent presence of multiple nodules in patients with HCC[5] provides a problem of differentiation between cancer of multifocal origin and different cancer foci originating from a single tumor through metastasis. This issue is important from a clinical viewpoint, since these two categories correspond to different stages of the disease, and therefore subsequent treatment and prognosis would also differ. However, clinical studies of this aspect have not been adequate up to now because of the difficulty involved in differential diagnosis of multiple cancers.

Pathological criteria, based mainly on macroscopic and histological observations, have been proposed for differential diagnosis of multiple HCC (1). Differentiation is relatively easy when nodules show different histology, but in cases with similar histology and/or when tumors are closely adjacent it becomes very complicated. In any event, the final diagnosis is left to the subjective judgment of a pathologist where objective evidence is lacking. This uncertainty has prevented the advance of clinical and basic studies of multiple HCC. Therefore, objective methods for the differential diagnosis of multiple cancers and experimental demonstration of the validity of existing pathological criteria are clearly desirable.

As an objective approach, some genetic methods for analysis of multifocal HCC have already been applied, but they have been far from satisfactory. One example is Southern blot examination of the integration pattern of hepatitis B virus in multiple HCC (2, 3) but this is applicable to only a small percentage of HCCs which harbor the virus (4).

In order to find a quicker and more reliable method for the differential diagnosis of multiple HCC, we focused on the mutation pattern of the *p53* gene. Since an accumulation of various genetic alterations has been demonstrated in carcinoma cells (5), we reasoned that genetic abnormalities in different nodules would probably differ in cases of multifocal origin. Mutations of the *p53* gene have been observed in 29–36% of single-nodule HCCs (6),[6] and a higher frequency would be expected in cases with multiple nodules.

In the present study we analyzed the *p53* mutation pattern in 58 nodules from 26 cases of HCC by the PCR-SSCP (7), a simple and sensitive method for detection of DNA mutations. We report here that analysis of the *p53* mutation pattern could be a useful marker for the differential diagnosis of multiple cancers.

MATERIALS AND METHODS

Patients and DNA Extraction. Fifty-eight samples of tumor tissue obtained from 26 patients who had undergone surgical treatment for multiple HCC at the National Cancer Center Hospital, Tokyo, were studied. Four cases each involved 3 nodules, and the remaining 22 cases had 2 nodules each. The main tumor nodule in each case, *i.e.*, the larger and/or less differentiated one, was named T1. In both case 2 and case 5, another tumor was obtained at a second operation, and this was an intrahepatic recurrent nodule and an abdominal lymph node metastasis, respectively (see Table 1). We classified the cases into 3 groups according to the pathological criteria (1, 2, 8, 9) shown in Fig. 1: multifocal (19 cases); metastatic (4 cases); undetermined (3 cases). DNA was extracted by digestion with proteinase K, extraction with phenol/chloroform, and precipitation with ethanol (10).

PCR-SSCP Analysis. Because 98% of *p53* gene mutations in different cancers have been found in exons 5–8 (11), we focused our study on these exons. The PCR-SSCP method described by Orita *et al.* (7) was used to detect the presence and pattern of *p53* gene mutation.

The oligonucleotide primers for amplification of exons 5–8 were designed based on the published sequence (12):

Exon 5:
5′GGAATTCCTCTTCCTGCAGTAC3′,
5′GGAATTCGCCCCAGCTGCTCACCATCG3′
Exon 6:
5′GGAATTCGATGGTGAGCAGCTGG3′,
5′GGAATCAGTTGCAAACCAGACCTCAGG3′
Exon 7:
5′GGAATTCTCCTAGGTTGGCTCTGAC3′,
5′GGAATTCCAAGTGGCTCCTGACCTGGA3′
Exon 8:
5′GGAATTCCCTATCCTGAGTAGTGGTAA3′,
5′GGAATTCCTGCTTGCTTACCTCG3′

One hundred ng of genomic DNA were subjected to 35 cycles of PCR (94°C, 55°C, 72°C for 0.5, 0.5, 1 min, respectively) in 10 μl of solution

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[2] Awardee of a Research Resident Fellowship.

[3] Supported by a Foreign Research Fellowship from the Foundation for Promotion of Cancer Research, Tokyo, Japan. Present address: Institute of Pathology, Verona University, Strada Le Grazie, 37134 Verona, Italy.

[4] To whom requests for reprints should be addressed.

[5] The abbreviations used are: HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism.

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Table 1 *p53 mutation pattern in multiple hepatocellular carcinomas*

Case	Nodule	Morphological Information: Diagnosis	Size (cm)	Differentiation	SSCP: Abnormal band	Exon	Sequencing: Codon	Nucleotide	Amino acid	Mutation pattern[a]
1	T1	Multifocal	1.9	Well	+	8	282	CGG→TGG	Arg→Trp	A-0
	T2		1.9	Well	−					
2	T1	Multifocal	6.5	Poor	+	7	241	TCC→GCC	Ser→Ala	A-B
	T2		5.5	Poor	+	7	249	AGG→TGG	Arg→Trp	
	R		2.2	Moderate	+	7	249	AGG→TGG	Arg→Trp	
3	T1	Multifocal	7.6	Moderate	+	5	176	TGC→AGC	Cys→Ser	A-0
	T2		2.4	Well	−					
4	T1	Multifocal	4.3	Poor	+	7	239	AAC→AGC	Asn→Ser	A-0
	T2		1.7	Well	−					
5	T1	Multifocal	2.0	Poor	+	7	236	TAC→TGC	Tyr→Cys	A-0
	T2		2.6	Well	−					
	LN		7.0	Poor	+	7	236	TAC→TGC	Tyr→Cys	
6	T1	Multifocal	2.7	Moderate	+	5	130	CTC→CGC	Leu→Arg	A-0
	T2		1.8	Moderate	−					
	T3		1.3	Well	−					
7	T1	Multifocal	3.8	Poor	+	7	248	CGG→CAG	Arg→Gln	A-0
	T2		3.1	Moderate	−					
	T3		1.8	Poor	−					
8	T1	Multifocal	4.5	Poor	+	5	157–159	7-base pair del	Frame shift	A-0
	T2		1.7	Well	−					
9	T1	Multifocal	3.0	Poor	+	7	237–243	16-base pair del	Frame shift	A-0
	T2		1.3	Moderate	−					
10	T1	Multifocal	5.2	Moderate	+	5	141	1-base pair del	Frame shift	A-B
	T2		3.6	Combined	+	7	249	AGG→AGT	Arg→Ser	
11	T1	Multifocal	2.4	Moderate	+	5	163	TAC→AAC	Tyr→Asn	A-0
	T2		2.8	Well	−					
12	T1	Metastatic	10.5	Moderate	+	7	245	GGC→TGC	Gly→Cys	A-A
	T2		0.8	Moderate	+	7	245	GGC→TGC	Gly→Cys	
13	T1	Metastatic	9.0	Moderate	+	7	242	TGC→TTC	Cys→Phe	A-A
	T2		4.5	Moderate	+	7	242	TGC→TTC	Cys→Phe	
14	T1	Metastatic	6.5	Poor	+	6	219	1-base pair del	Frame shift	A-A
	T2		1.2	Poor	+	6	219	1-base pair del	Frame shift	
15	T1	Undetermined	4.5	Poor	+	8	273	CGT→TGT	Arg→Cys	A-A
	T2		3.9	Poor	+	8	273	CGT→TGT	Arg→Cys	
16	T1	Undetermined	4.0	Poor	+	7	249	AGG→TGG	Arg→Trp	A-A
	T2		0.9	Moderate	+	7	249	AGG→TGG	Arg→Trp	
17	T1	Undetermined	4.5	Poor	+	8	278	CCT→CGT	Pro→Arg	A-A
	T2		1.8	Moderate	+	8	278	CCT→CGT	Pro→Arg	

[a] A-A, same mutation in different nodules; A-B, different mutations in different nodules; A-0, mutation in one of the nodules; R, recurrent liver tumor resected 8 months after the previous operation; LN, abdominal lymph node metastasis found 2 years after the previous operation; Combined, combined histology of cholangiocellular and hepatocellular carcinoma.

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containing 0.25 pmol each of 5′-end-labeled primers (13), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 20 μM deoxynucleotide triphosphatess, and 0.5 unit of Taq polymerase (Perkin Elmer/Cetus, Norwalk, CT).

The PCR products were diluted 1:100 in loading solution (96% formamide, 20 mM EDTA, 0.05% xylene cyanol, and bromophenol blue), denatured at 85°C for 3 min, and applied (1 μl/lane) to 6% polyacrylamide/Tris-borate EDTA gel with and without 5% (v/v) glycerol. Electrophoresis was performed at 30 W for 2.5–5 h at room temperature with vigorous air cooling. The gel was dried on filter paper and exposed to X-ray film (Kodak XRP-1) at room temperature for 2–12 h.

Direct DNA Sequencing. Abnormal bands detected by SSCP analysis were eluted from the gel and amplified by 55 cycles of asymmetrical (20:1 primer ratio) PCR (14). The single strand products were purified in a Centricon 30 microconcentrator (Amicon, Beverly, MA) and subjected to sequencing using a 7-deaza-GTP Sequenase version 2 kit (United States Biochemicals, Cleveland, OH) with 5′-end-labeled primers. The sequencing primers for sense (-s) and antisense (-a) of each exon were as follows:

5-s 5′TCTTCCTGCAGTACTCCCCT3′,
5-a 5′AGCTGCTCACCATCGCTAT3′,
6-s 5′CACTGATTGCTCTTAGGT3′,
6-a 5′TGCAAACCAGACCTCAGG3′,

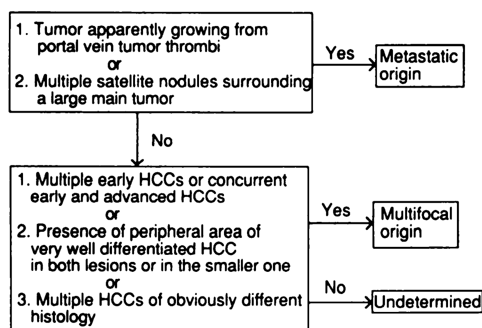

Fig. 1. Morphological criteria for differential diagnosis of multiple hepatocellular carcinoma. These criteria are based on the following findings; *1*, HCC metastasizes mainly through the portal vein (1); *2*, HCC at an early stage does not show metastasis (1, 8); *3*, primary HCC often shows morphological evidence of well differentiated peripheral lesions (2, 9).

7-s 5′TAGGTTGGCTCTGACTGT3′,
7-a 5′GTGGCTCCTGACCTGGAGTC3′,
8-s 5′ATCCTGAGTAGTGGTAATCT3′,
8-a 5′GCTTGCTTACCTCGCTTAGT3′.

The products were analyzed in 8% polyacrylamide/Tris-borate EDTA gel containing 5 M urea.

RESULTS

Seventeen of the 26 (65%) multiple HCC cases showed abnormally shifted bands in at least one of their nodules upon SSCP analysis of the *p53* gene. The remaining 9 cases were negative. All of the abnormal bands were analyzed further by direct DNA sequencing, and the results are summarized in Table 1. The positive cases were classified into three groups according to the pattern of mutation between nodules as follows (representative cases are presented in Fig. 2): group 1, A-A pattern: *same* mutation in different nodules (6 cases); group 2, A-B pattern: *different* mutations in different nodules (2 cases); group 3, A-0 pattern: mutation in *one* of the nodules (9 cases).

The range over which mutations occurred was spread over 456 base pairs of codons 130–282. It is worth noting that the *p53* mutations obtained differed from case to case. Only cases 16 and 2 (nodule T2) displayed the same mutation, although case 2 also possessed a different mutation in nodule T1.

Of the two samples obtained at a second operation, the recurrent hepatic nodule in case 2 showed the same mutation as that of nodule T2 and the lymph node metastasis in case 5 displayed the same mutation as that of nodule T1 (see Table 1).

A comparison between the pathological diagnosis and the *p53* mutation pattern is as follows. In 19 cases of pathologically multifocal HCC, *p53* mutation was observed in 11 (58%). The A-B pattern was detected in 2 cases, and the other 9 cases showed the A-0 pattern, but none of multifocal cases showed the A-A pattern. Three (75%) of 4 pathologically metastatic cases were positive for the *p53* mutation, and their patterns were all A-A. All 3 of the pathologically undetermined cases were also informative in the present analysis, showing the A-A pattern.

DISCUSSION

The main concept that we propose here is the application of gene mutation to provide a marker of each tumor nodules. *p53* gene mutation analysis of cases of multiple HCC was demonstrated to be a good method for objective differentiation between multifocal and metastatic multiple tumor nodules. In fact, as many as 65% (17 of 26) of multiple HCCs showed the *p53* gene mutation in at least one nodule. The mutations differed from case to case, showing a large diversity that served as a useful marker for each tumor nodule.

Our analysis showed the existence of three different patterns of *p53* mutation in multiple HCC. Patterns A-B and A-0 indicating heterogeneity of the *p53* mutation between nodules, with regard to type and presence, respectively, suggest different origins from different cellular clones. Therefore, the cases showing these two genetic patterns could be diagnosed as multifocal in origin. On the other hand, nodules showing the same genetic abnormality in cases with an A-A pattern are likely to be derived from the same clone, and thus metastatic in origin.

However, a few further possibilities should be considered. One is that a case with metastatic nodules from a *p53* mutation-negative main tumor could show an A-0 pattern when one of the nodules acquires the mutation through later progression. Although *p53* abnormalities are regarded as a late event in cancer progression, they usually occur earlier than the formation of metastasis in a genetic model for colorectal tumorigenesis (15). Moreover, the mutations in cases with an A-0 pattern were always observed in T1 (primary, larger and/or less differentiated tumors) and never in T2 or T3 (secondary, smaller and/or less advanced tumors), and the possibility that T2 or T3 may have arisen as metastatic tumors from T1 should be very small. None of 11 T2 or T3 tumors with A-0 patterns presented portal vein tumor thrombi while HCC metastasize mainly through portal veins. In addition, 6 of these 11 tumors were well differntiated including 2 early HCCs which rarely show metastasis (1, 8). From these findings, the possibility that T2 giving rise to the T1 should be very small again. Therefore, we considered that the genetic heterogeneity found in cases with an A-0 pattern is mainly due to the multifocal origin of the different nodules. Another possibility is that cases of multifocal HCC could show an A-A pattern by possessing the same mutation by chance. However, this possibility will be extremely low at least in Japanese cases of HCC, since in this investigation and that of Murakami *et al.* (6) all but one of the mutations differed among the examined cases. It was recently reported that *p53* mutations of HCC in certain areas of China and South Africa, where there is heavy exposure to aflatoxin B_1, were almost all localized to codon 249, but this could be an exceptional phenomenon related to the special etiology (16, 17).

The usefulness of our approach is supported also by the fact that it was possible to determine a metastatic or multifocal origin in three of four cases where morphological analysis was not helpful. Because these cases showed A-A patterns, we were able to recognize their metastatic origin. Another notable result of our study was that the origin of recurrent tumors and metastasis was clarified through analysis of the *p53* mutation pattern. That is, the same mutation of R as that of T2 in case 2 and of LN with that of T1 in case 5 suggested their metastatic origin from one of the nodules of the respective primary HCCs.

Furthermore, by comparing the differential diagnosis of multiple HCCs based on morphological information and the results of genetic analysis, no discrepancy was observed among the comparable cases, *i.e.*, those positive for the *p53* mutation. This lends objective support to the validity of the pathological criteria used at the National Cancer Center Hospital, Tokyo (shown in Fig. 1) for the differential diagnosis of multiple HCCs. However, the clinical need for a preoperative (pretherapeutic) objective diagnosis of a large number of cases can be efficiently met by genetic analysis of *p53* mutations in biopsy

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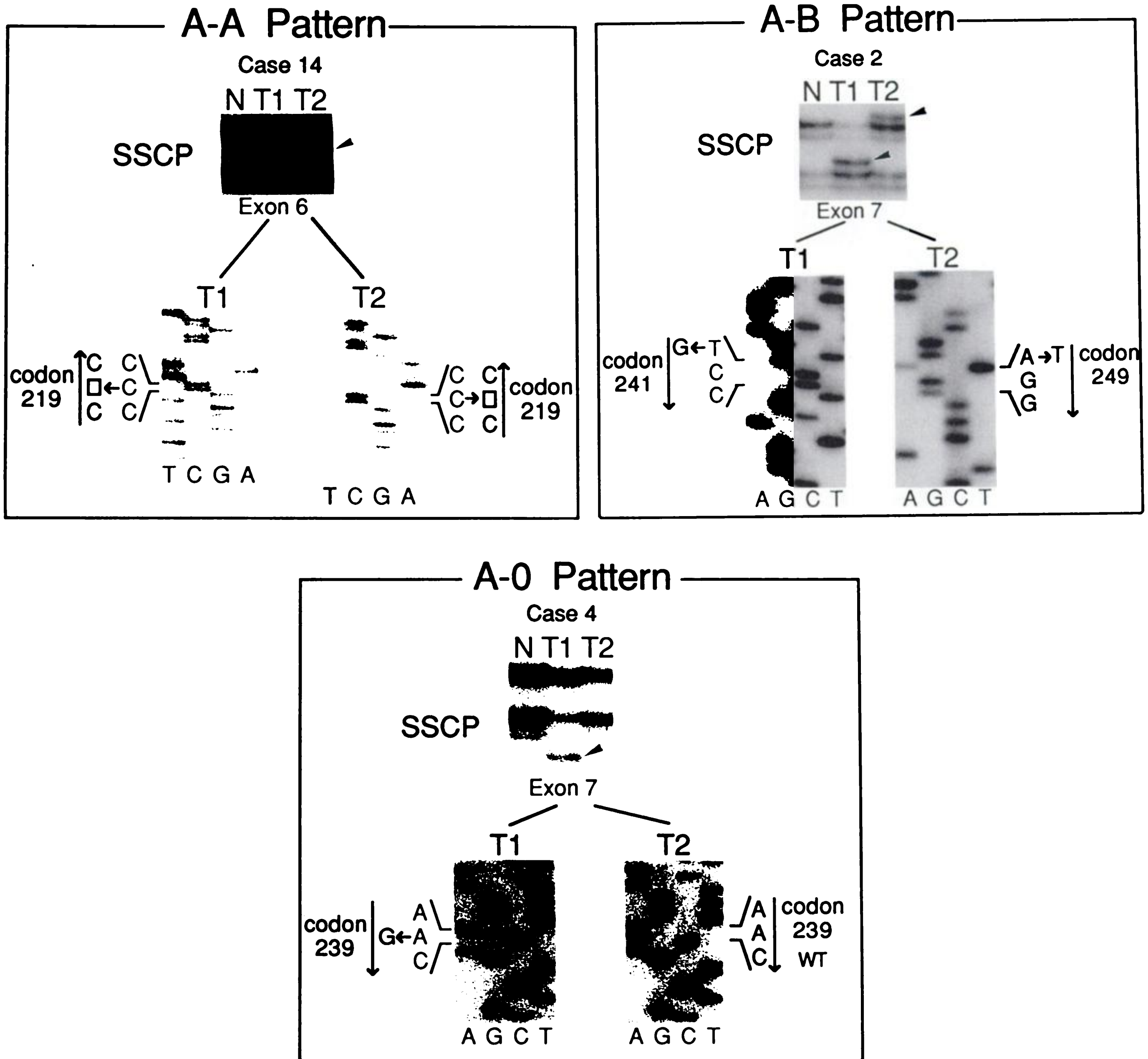

Fig. 2. Cases representative of the three mutation patterns of the *p53* gene in multiple hepatocellular carcinoma. For SSCP analysis DNAs from different tumors of the same patient (T1 and T2) and a normal control (*N*) were analyzed for abnormalities of *p53* gene exons. Cases were considered positive for mutation when bands different from that of normal controls were observed (*arrowhead*). For DNA sequencing analysis (*lower half* of each pattern panel) the sequence obtained from the abnormal bands evident on SSCP analysis are shown. Each sequence is shown 5' (*bottom*) to 3' (*top*) for the noncoding strand for exon 7 and the coding strand for exon 6. The codons at which the mutation occurs are indicated. For exon 7, the indicated letters are the translated coding sequence obtained from the results. A-A pattern, different nodules with the same mutation; A-B pattern, different nodules with different mutations; A-0 pattern, only one of the nodules has a mutation.

material obtained by fine-needle aspiration.

The PCR-SSCP method has up to now been the easiest and quickest method for detecting point mutations and allows immediate comparisons to be made among different tumors without sequencing. In the present study, although we sequenced all the samples that were positive upon SSCP analysis, a simpler approach involving only PCR-SSCP would be more than adequate for determining whether the gene mutations are different or the same in different samples.

The present approach should prove applicable to differential diagnosis of other multiple cancers such as those of the colon, lung, and bladder, in which *p53* abnormalities are also observed frequently (11). However, use of *p53* may not be decisive, since *p53* abnormalities are not present in every case and are considered to be a relatively late event in cancer progression (6, 15).[6] When the genetic alterations involved in the early stage of carcinogenesis are elucidated, analysis of these genes may yield much accurate information. Finally, we expect that clinical studies of multiple cancer will advance, aided by the objective diagnosis provided by the present method, in conjunction with existing pathological criteria.

REFERENCES

1. Kanai, T., Hirohoshi, S., and Shimosato, Y. Pathology of small hepatocellular carcinoma (a proposal for a new gross classification). Cancer (Phila.), *60:* 810–819, 1987.
2. Tsuda, H., Hirohashi, S., Shimosato, Y., Terada, M., and Hasegawa, H. Clonal origin of atypical adenomatous hyperplasia of the liver and clonal

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identity with hepatocellular carcinoma. Gastroenterology, *95:* 1664–1666, 1988.
3. Sakamoto, M., Hirohashi, S., Tsuda, H., Shimosato, Y., Makuuchi, M., and Hosoda, Y. Multicentric independent development of hepatocellular carcinoma revealed by analysis of hepatitis B virus integration pattern. Am. J. Surg. Pathol., *13:* 1064–1067, 1989.
4. Okuda, K., Fujimoto, I., Hanai, A., and Urano, Y. Changing incidence of hepatocellular carcinoma in Japan. Cancer Res., *47:* 4967–4972, 1987.
5. Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M. M., and Bos, J. L. Genetic alternations during colorectal-tumor development. N. Engl. J. Med., *319:* 525–532, 1988.
6. Murakami, Y., Hayashi, K., Hirohashi, S., and Sekiya, T. Aberrations of the tumor suppressor *p53* and retinoblastoma genes in human hepatocellular carcinomas. Cancer Res., *51:* 5520–5525, 1991.
7. Orita, M., Suzuki, Y., and Hayashi, K. Rapid and sensitive detection of point mutation and DNA polymorphisms using the polymerase chain reaction. Genomics, *5:* 874–879, 1989.
8. Sakamoto, M., Hirohashi, S., and Shimosato, Y. Early stages of multiple hepatocarcinogenesis: adenomatous hyperplasia and early hepatocellular carcinoma. Hum. Pathol., *22:* 172–178, 1991.
9. Kenmochi, K., Sugihara, S., and Kojiro, M. Relationship of histologic grade of hepatocellular carcinoma (HCC) to tumor size, and demonstration of tumor cells of multiple different grades in single small HCC. Liver, *7:* 18–26, 1987.
10. Sambrook, J., Fritsch, E. F., and Maniatis, T. (eds.). Molecular Cloning: A Laboratory Manual, Ed. 2. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
11. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. *p53* mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.
12. Buchman, V. L., Chumakov, P. M., Ninkina, N. N., Samarina, O. P., and Georgiev, G. P. A variation in the structure of the protein-coding region of the human *p53* gene. Gene, *70:* 245–252, 1988.
13. Hayashi, K., Orita, M., and Sekiya, T. Use of labeled primer in polymerase chain reaction (LP-PCR) for a rapid detection of the product. Nucleic Acids Res., *17:* 3605, 1989.
14. Suzuki, Y., Sekiya, T., and Hayashi, K. Allele-specific polymerase chain reaction: a method for amplification and sequence determination of a single component among a mixture of sequence variants. Anal. Biochem., *192:* 82–84, 1991.
15. Fearon, E. R., and Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell, *61:* 759–767, 1990.
16. Hsu, I. C., Metcalf, R. A., Sun, T., Welsh, J. A., Wang, N. J., and Harris, C. C. Mutational hotspot in the *p53* gene in human hepatocellular carcinomas. Nature (Lond.), *350:* 427–428, 1991.
17. Bressac, B., Kew, M., Wands, J., and Ozturk, M. Selective G to T mutation of *p53* gene in hepatocellular carcinoma from southern Africa. Nature (Lond.), *350:* 429–431, 1991.

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