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Somatic Mutations of the *APC* Gene in Sporadic Hepatoblastomas[1]

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ABSTRACT

Hepatoblastoma is a rare hepatic malignancy that occurs in children with an average age of 2 or 3 years and is known to be one of the extracolonic manifestations of familial adenomatous polyposis. Only a single hepatoblastoma with a germ-line mutation of the adenomatous polyposis coli (*APC*) gene has been reported thus far. To elucidate the possible roles of *APC* gene alterations in sporadic hepatoblastomas, we examined loss of heterozygosity (LOH) at the *APC* and *MCC* loci and performed a sequencing analysis of a part of the *APC* gene, including the mutation cluster region, in 13 hepatoblastomas of non-familial adenomatous polyposis patients. LOH at the *APC* and/or *MCC* loci was observed in four of seven (57%) informative cases. Of the 13 cases, somatic mutations were detected in 8 (61.5%), with 9 (69%) cases showing genetic alterations in the *APC* gene as LOH or somatic mutations. Two cases demonstrated double mutations. Furthermore, the nature of the somatic mutations observed in the present study was unusual because 9 of the 10 mutations were missense, with only 1 case featuring a frame-shift mutation due to an insertion. Previous reports have described almost all (>90%) mutations of the *APC* gene in colorectal tumors to result in a truncated APC protein due to either frame-shift or nonsense mutations. These findings suggest that a mutation of the *APC* gene may play an important role in the genesis of sporadic hepatoblastomas, and the mechanisms of *APC* gene alteration may be different from those reported previously for colorectal tumors.

INTRODUCTION

Hepatoblastoma is a typical embryonic tumor that occurs in children with an average age of 2 or 3 years (1). Although genetic analysis has progressed to the extent of explaining other childhood malignancies such as retinoblastoma and Wilms' tumor, the evidence of genetic alteration in hepatoblastomas is limited. Several investigators have examined the status of the *p53* gene (2, 3) and established that a hot spot at codon 157 is closely associated with tumorigenesis (3). However, the possibility that the other genes may play some roles in hepatoblastoma development remains to be elucidated.

It is well known that hepatoblastomas occur in patients who have a family history of polyposis coli (4–8). The occurrence rate is estimated to be about 1000–2000 times higher than that of sporadic cases without such a family history (6–8). Furthermore, some patients with a history of hepatoblastomas in childhood have been found to suffer adenomatous colon polyposis in later life (6). Thus, a close relation between hepatoblastomas and FAP[3] is implied. Since the *APC* gene was first cloned from linkage analysis in 1991 (9, 10), germ-line mutations have been observed in FAP patients, and somatic mutations have been detected in colorectal and gastric tumors of not only FAP patients but also of non-FAP patients (11–18). However, only a single hepatoblastoma with a germ-line mutation of the *APC* gene has been reported thus far (19).

To clarify the role of *APC* gene mutations in the development of sporadic hepatoblastomas, we have investigated their occurrence and the LOH of the *APC* and *MCC* loci in 13 non-FAP patients with hepatoblastomas. Here we report evidence that somatic mutations of the *APC* gene with LOH at the *APC* and/or *MCC* loci occur at high frequency in sporadic hepatoblastomas.

MATERIALS AND METHODS

Materials. A total of 13 cases of hepatoblastoma were analyzed. Data on each patient's age, sex, and histological classification according to Ishak and Glunz (1) are shown in Table 1. Samples from cases 1–8 were surgically resected in Tokyo University Hospital. Materials from cases 9–13 were taken at autopsy (performed 2–4 h after death) at the Department of Pathology, University of Tokyo. The age of patients ranged from 4 months to 10 years, with an average age of 3 years and 3 months. Of the 13 patients, 9 were male. In terms of histological classification (1), eight of the hepatoblastoma cases were the purely epithelial type, the remainder were the mixed epithelial and mesenchymal type (Fig. 1). All the cases reported here were Japanese and sporadic in nature, with no definite family history of polyposis in the colorectum.

DNA Preparation. Twelve (cases 1–7 and 9–13) of the hepatoblastoma samples were obtained from formalin-fixed, paraffin-embedded liver tissues as described previously (20). Briefly, paraffin blocks were sectioned serially at 15 μm and were attached to glass slides, were stained alternately with H&E, and then tumor and nontumorous parts were independently excised from 10 slides of each specimen under a microscope. To avoid contamination, microtome blades were changed between samples. Paraffin was eliminated by three extractions with xylene, followed by immersion in ethanol and drying and digestion in a 500-μl lysis buffer [50 mM Tris-HCl (pH 8.0), containing proteinase K to a final concentration of 0.5 mg/ml] at 40°C for 24 h. After phenol-chloroform extraction, genomic DNA was precipitated with ethanol. One sample (case 8) obtained at surgery was immediately frozen in liquid nitrogen and stored at −80°C until use. Genomic DNA from fresh-frozen tissue was prepared by proteinase K-phenol-chloroform extraction. All possible precautions were taken to avoid contamination. Both tumors and corresponding normal parts were obtained in all cases.

LOH Analyses. LOH analysis was performed as described previously (21). Briefly, for the *APC* gene, primers flanking a *Rsa*I RFLP within exon 11 were synthesized. Primer sequences were 5′-GGACTACAGGCCATTGCAGAA-3′ and 5′-GGCTACATCTCCAAAAGTCAA-3′. For the *MCC* gene, primers flanking a variable insertion polymorphism within exon 10 were synthesized. Primer sequences were 5′-TACGAATCCAATGCCACA-3′ and 5′-CTGAAGTAGCTCCAAACA-3′. Genomic DNA (0.5 μg) was dissolved in a total volume of 50 μl of solution containing 1 × PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin (w/v), 0.2 mM of each deoxynucleotide triphosphate, 1 μM of each primer, and 2.5 units of *Taq* polymerase]. Templates were denatured for 5 min at 92°C, followed by 40 cycles of PCR with incubations of 1 min at 55°C, 2 min at 72°C, and 1 min at 92°C. For the *APC* analysis, the 133-bp PCR products were digested with 20 units of *Rsa*I (Toyobo, Osaka, Japan). For *MCC*, PCR amplified a product either 79 or 93 bp long, depending upon the absence or presence of an insertion. Therefore, no restriction enzyme digestion was necessary. PCR products were then electrophoresed on a 3% agarose gel and were stained with ethidium bromide. LOH was defined as a visible change in the allele:allele ratio in tumor DNA relative to the ratio in corresponding normal DNA. The bands were scored by an observer blind to patients' histories.

Sequencing Analysis of the *APC* Gene. The 5′ site of exon 15 (codons 1268–1578) was divided into 13 segments, which included the mutation cluster region (codons 1286–1513; Ref. 13) known from colorectal carcino-

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[3] The abbreviations used are: FAP, familial adenomatous polyposis; APC, adenomatous polyposis coli; LOH, loss of heterozygosity.

Table 1 *Somatic mutations of APC and LOH at APC and MCC in hepatoblastomas*

Case	Age/sex[a]	Histological type[b]	*APC* mutations			LOH[c]	
			Codon	Nucleotide change	Effect of mutations	*APC* exon 11	*MCC* exon 10
1	5y7m/M	Epi	1395	AGT to TGT/AGT	Ser to Cys	NI	NI
			1534	GGG to AGG/GGG	Gly to Arg		
2	5m/M	Mixed	1395	AGT to TGT/AGT	Ser to Cys	HET	HET
3	6y5m/M	Epi	1326	GTG to ATG	Val to Met	LOH	LOH
4	10y/M	Epi	1326	GTG to ATG	Val to Met	LOH	LOH
5	2y/M	Mixed	1395	AGT to TGT/AGT	Ser to Cys	HET	HET
6	1y/M	Mixed	WT[d]			LOH	NI
7	7y/M	Epi	WT			NI	NI
8	7m/M	Epi	1330	CCT to CCCT/CCT	C insertion	NI	NI
9	2y4m/F	Mixed	WT			NI	NI
10	3y/F	Epi	1306	GAA to AAA	Glu to Lys	LOH	LOH
11	2y6m/F	Mixed	WT			HET	HET
12	4m/M	Epi	1319	CCT to ACT	Pro to Thr	NI	NI
			1321	AGC to AAC	Ser to Asn		
13	1y2m/F	Epi	WT			NI	NI

[a] y, years; m, month.
[b] Epi, epithelial; Mixed, mixed epithelial and mesenchymal.
[c] NI, not informative; HET, retained heterozygosity.
[d] WT, wild type.

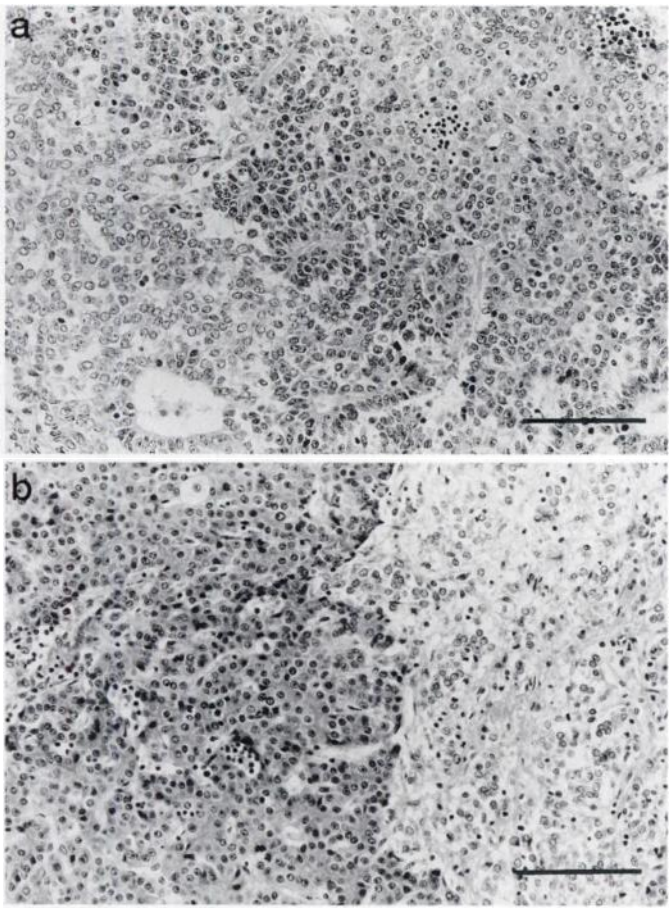

Fig. 1. Typical histology of epithelial-type (*a*, case 1) and mixed epithelial and mesenchymal-type (*b*, case 2) hepatoblastoma. Note that both types of hepatoblastoma contain a relatively large number of extramedullary hematopoietic cells. H&E staining. *Scale bar*, 100 μm.

mas; these segments were amplified by PCR, using primer sets as follows: segment 1, 5′GACTTATTGTGTAGAAGATACT3′ and 5′GCAGGGTATTAGCAGAATCT3′; segment 2, 5′GAAATAGGATGTAATCAGACG3′ and 5′TGGTTCTAGGGTGCTGTGA3′; segment 3, 5′TCAGCTGAAGATCCTGTGAGC3′ and 5′CTTTGTGCCTGGCGTATTC3′; segment 4, 5′AGCAGACTGCAGGGTTCTA3′ and 5′GAGTGGGGTCTCCTGAAC3′; segment 5, 5′CCAAAAGTGGTGCTCAGAC3′ and 5′ATGGTTCACTCTGAACGGA3′; segment 6, 5′TCTGTCAGTTCACTTGATAGT3′ and 5′GGTTTGTCCAGGGCTATCT3′; segment 7, 5′GGTAAGTGGCATTATAAGCC3′ and 5′AGGTACTTCTCGCTTGGTTT3′; segment 8, 5′CCATGCCACCAGCAGAAG3′ and 5′TCTTTTCAGCAGTAGGTGCT3′; segment 9, 5′AAACCAAGCGAGAAGTAGCT3′ and 5′TATCAGCTGGAAGAACC3′; segment 10, 5′AGCACCTACTGAAAAGA3′ and 5′AAATCCATCTGGAGTACTTTC3′; segment 11, 5′GGTTCTTCCAGATGCTGATA3′ and 5′AGGCATTATTCTTAATTCCACA3′; segment 12, 5′CCTGAGTGCTCTGAGCCT3′ and 5′CTCTTGGTTTTCATTTGATTCT3′; and segment 13, 5′AAATGACAATGGGAATGAAAC3′ and 5′TTGGCATGGCAGAAATAAATAC3′.

PCR was performed with 40 cycles, under the same conditions as described in "LOH Analyses." The amplified PCR products were purified by electrophoresis, using low-melting-point agarose. The purified products were then subcloned into the *Eco*RV site of a T-tailed pBluescript II SK(−) (Stratagene, La Jolla, CA) as described previously (22). The sequences of DNA from mixed recombinant colonies (at least 50 subclones) were determined for both of the strands by the dideoxyribonucleotide chain termination method, using a T7 sequencing kit (Pharmacia). When a mutation was identified, both the PCR and sequencing reactions were repeated to confirm the original results.

RESULTS

LOH Analysis. Normal DNA from heterozygous patients showed three bands at the *APC* exon 11 locus: a 133-bp band from the allele lacking the *Rsa*I restriction enzyme site and two cut bands, 85 bp and 48 bp, representing the allele that contained the site. Heterozygous patients showed two bands at the *MCC* exon 10 locus: a 79-bp band representing one allele and a 93-bp band representing the other. Several examples of *APC* LOH and *MCC* LOH are shown in Fig. 2. Data for individual cases are displayed in Table 1. At the *APC* locus, 7 of 13 cases were informative, and 4 of 7 demonstrated LOH. At the *MCC* locus, 6 of 13 cases were informative, and 3 of 6 showed LOH. Therefore, four cases were heterozygous for at least one locus, and LOH at *APC* and/or *MCC* was detected in 57% (four of seven) of the cases.

Mutation of the *APC* Gene. Of the 13 hepatoblastomas, 8 were found to have mutations in the *APC* gene examined, as shown in Table 1. All but one (case 8) of the cases with alterations in the *APC* gene had point mutations, causing amino acid substitutions by missense. No nonsense mutation was observed. One case (case 8) showed a single base insertion from CCT to CCCT at codon 1330. This change created a new stop codon immediately downstream due to the frame shift. Four of nine missense mutations were transversion-type mutations such as A to T (three cases) or C to A (one case), and the other five mutations were G to A transitions. Two cases (cases 1 and 12) had double mutations. As shown in Table 1 and Fig. 3, the mutations

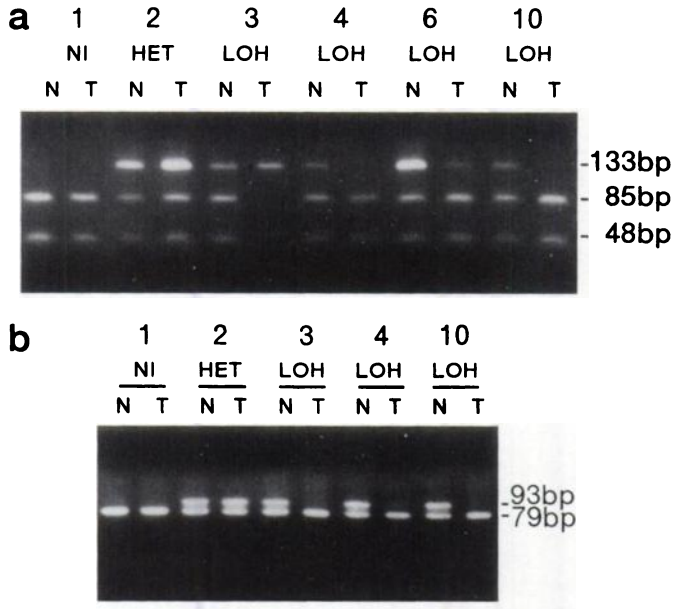

Fig. 2. LOH analysis of *APC* exon 11 (*a*) and *MCC* exon 10 (*b*). Cases 3, 4, 6, and 10 showed LOH for the *APC* gene (*a*); and cases 3, 4, and 10 exhibited LOH for the *MCC* gene (*b*). *NI*, not informative; *HET*, retained heterozygosity; *N*, normal; *T*, tumor portion. *Numbers (top)*, case numbers.

of four cases were expressed alone without the wild type, whereas in the remaining four cases, coexistent mutant and wild types were noted. The same mutations were observed at codon 1395 in three cases (cases 1, 2, and 5) and at codon 1326 in two cases (cases 3 and 4). No mutation was detected in DNA extracted from nontumorous parts of the specimens.

DISCUSSION

This is the first report to describe a high frequency of *APC* gene alterations in sporadic hepatoblastomas. We have found that 8 of 13 cases (61.5%) showed point mutations in the *APC* gene. All had the wild type in nontumorous parts, suggesting the mutations were somatic. LOH analysis resulted in four of seven (57%) informative cases showing LOH at *APC* and/or *MCC* loci. Thus, a total of 9 of the 13 cases examined (69%) demonstrated alterations of the *APC* gene, either mutations or LOH. Because the region of the *APC* gene examined in the present study covers only about 10% of the entire coding region, albeit that containing the mutation cluster region in which about 70% of the mutations were detected in previous studies (12–14), the true incidence might be much higher than that observed. Combining the results of LOH and mutational analysis, one allele was lost and the remaining allele showed point mutations in four cases. Furthermore, although one case (case 12) was not informative in LOH analysis, the sequence result again shows only the mutant band. The facts are compatible with Knudson's two-hit theory (23), and the *APC* gene alterations may play an important role in genesis of hepatoblastomas.

Epidemiological studies have suggested that hepatoblastomas occur frequently in families with polyposis coli, with an occurrence rate estimated about 1000–2000 times higher than that of sporadic cases (6–8). The *APC* gene was cloned recently, but there has only been a single previous report concerning the relation between alteration of the *APC* gene and sporadic hepatoblastomas. Kurahashi *et al.* (19) examined mutations of the *APC* gene in 11 cases and found one with a germ-line mutation, a G to T transversion at the splice-acceptor site of the intron 3-exon 4 junction, causing frameshift and somatic allele loss. The other cases were reported to have no alterations. It is

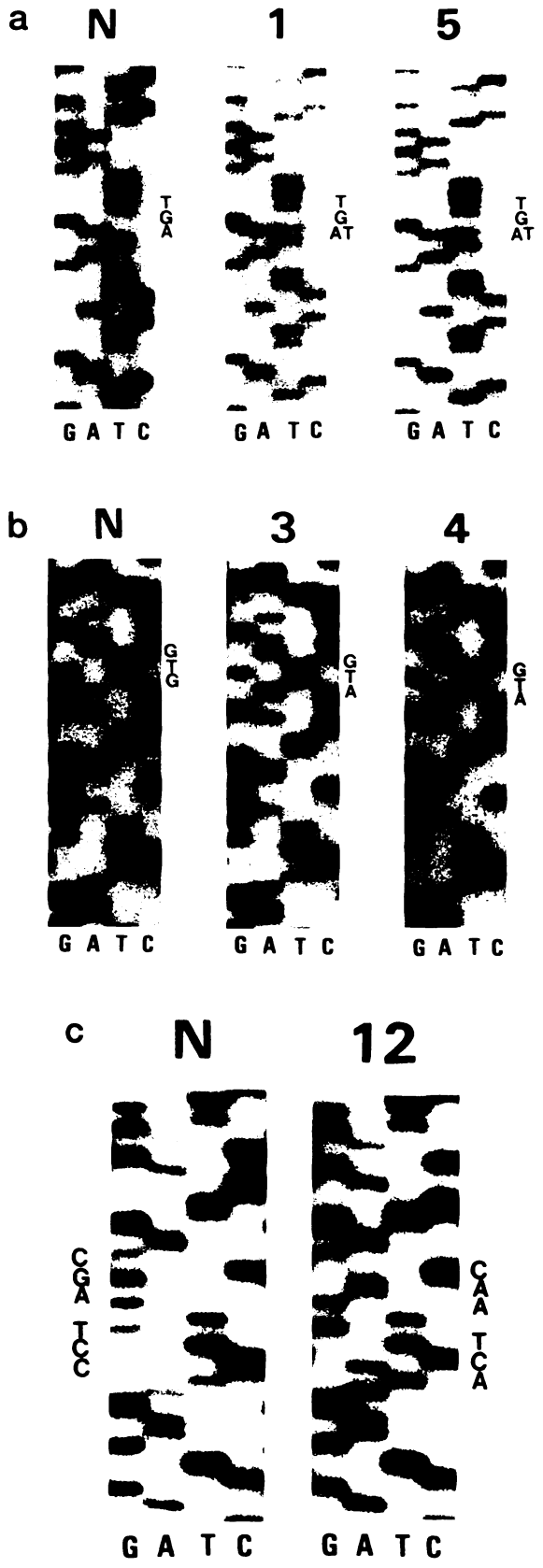

Fig. 3. Sequencing analysis of the *APC* gene. Tumor portions of cases 1 and 5 contain AGT to TGT/AGT mutations at codon 1395 (*a*); cases 3 and 4 show GTG to ATG mutations at codon 1326 (*b*); case 12 exhibits double mutations of CCT to ACT and AGC to AAC at codons 1319 and 1321, respectively. *N*, normal; *1, 3, 4, 5,* and *12*, tumor portions of cases 1, 3, 4, 5, and 12, respectively.

difficult to explain the difference with our results. However, we can speculate that differences in methodology might be important. We extracted DNA from specimens on glass slides under a microscope and sequenced all the cases, whereas Kurahashi *et al.* extracted DNA from tumor masses and performed screening with a RNase protection assay before sequencing. Because hepatoblastomas contain relatively large numbers of normal cells such as extramedullary hematopoietic cells (Refs. 1 and 3; Fig. 1), it is very difficult to detect LOH or mutations when DNA is extracted from the tumor as a mass. It is far more feasible when tumor cells are more specifically collected as performed in the present study. Although the RNase protection assay is a generally accepted approach for screening *APC* mutations, it detects at most 70% of mutations (12, 13). PCR amplification, cloning, and DNA sequencing processes adopted for the detection of APC mutations are more efficient.

None of the patients examined in this study had any family history of polyposis coli. Because the *APC* gene is extremely large, and the DNA samples in our materials were mainly extracted from paraffin blocks, we could not examine the entire coding regions or introns. Therefore, we cannot completely rule out that there might be a few patients with a new germ-line mutation in this study. Considering that some non-FAP patients who had a history of hepatoblastomas at a young age later suffered from APC (6), patients with mutations of the *APC* gene must be closely followed up for early detection of colorectal polyposis and cancer.

The mutated codons reported here have been found in earlier studies, mainly in colorectal tumors (14, 18). However, the mutation pattern in the present study is unusual. Although small differences among examined organs have been described, the most common types of alteration (>90%) in several types of cancers and adenomas are truncated-type mutations (such as frame shifts due to insertion or deletion) and nonsense mutations (leading to a stop codon). However, we found seven of eight cases of hepatoblastoma to have missense mutations, with only one case showing frame-shift mutation due to a single base insertion causing a stop codon immediately downstream. Although it is uncertain whether the missense mutations significantly affect the biological activity of the APC protein, we speculate that they might play an important role in development of colorectal cancers as well as hepatoblastomas. Although the numbers are small, missense mutations of the *APC* gene have been observed not only in the germ line of FAP patients but also as somatic mutations of FAP and non-FAP patients (12–18). A single amino acid change resulting from a missense mutation might influence the β-catenin binding activity of the APC protein that has been implicated in its function (24–27). Certain COOH-terminal regions might be important in the genesis of sporadic hepatoblastomas. Polakis (24) has suggested that β-catenin binding sites are also present in the COOH-terminal region (except for the publishing binding site). Additional studies are necessary to clarify this issue.

As shown in "LOH Analysis," the LOH events seem to be regional and always affect *APC* and, whenever informative, *MCC*. Although we could not determine the somatic mutations of *MCC* in this study, it remains possible that *MCC* also might be important in development of hepatoblastomas.

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