Bi-allelic Inactivation of the APC Gene in Hepatoblastoma

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ABSTRACT

Familial adenomatous polyposis (FAP) is an inherited disorder caused by germline mutation of the adenomatous polyposis coli (APC) gene. Increased risk of hepatoblastoma (HBL) in FAP kindreds has been reported. To determine whether inactivation of the APC gene plays a role in development of HBL, 13 sporadic infantile hepatic tumors were analyzed for genetic alterations in the APC gene. A PCR-mediated RNase protection analysis was performed to detect subtle genetic alterations in the mutation cluster region and in exons 3 and 4 of the APC gene. The results showed that a G to T transversion at the splice acceptor site of the intron 3-exon 4 junction had occurred in one HBL. Sequence analysis of normal tissue of the patient proved the mutation to be germinal. Southern blot analysis at the APC locus revealed that the tumor had lost the opposite allele and was isodisomic at this locus. RNA analysis indicated that the tumor contained only the small APC transcript, from which exon 4 was entirely absent. Since abnormal splicing causes termination due to frameshift, it was hypothesized that only the truncated APC protein was expressed in this tumor. These findings suggest that inactivation of the APC gene is closely related to tumorigenesis of HBLs in FAP patients.

INTRODUCTION

HBL is one of the most common embryonal tumors in infancy. These tumors occur generally within the first 2 years of life and are associated with an overall mortality rate of more than 75% (1). In spite of intensive treatment with multiantigen chemotherapy and total resection, children respond poorly. Tumorigenesis of HBL at the molecular level has not been clarified yet. Few cytogenetic studies have been published, although nonrandom abnormalities affecting chromosomes 2 and 20 have been identified (2, 3). HBL as well as Wilms’ tumor often develops in patients with Beckwith-Wiedemann syndrome, the genetic locus of which has been localized at 11p15 (4). Moreover, frequent LOH at 11p15 in HBL and overexpression of IGFI in an HBL cell line have been observed, suggesting that the IGFI gene can be considered a candidate as one responsible for HBL as well as for Wilms’ tumor (5, 6). However, biallelic expression due to relaxation of genomic imprinting is not observed in HBL, while the involvement of the IGFI gene in the development of HBL has not yet been convincingly demonstrated (7).

FAP is an autosomal dominant disorder characterized by the development of hundreds or thousands of colorectal adenomas during adolescence and young adulthood. If prophylactic colectomy is not performed, colorectal cancer will develop in virtually all affected individuals before the fifth decade of life. The disorder is caused by inherited germline mutation in a putative tumor suppressor gene, APC (8, 9). In the colorectal adenomas and cancers occurring in FAP patients, the remaining allele of the APC gene has been somatically inactivated by chromosomal deletion or point mutations (10). Even in sporadic colorectal cancer, the APC gene is also inactivated biallelically by two somatic mutations (11). Occurrence of infantile hepatic tumors in FAP patients has been reported often. Interestingly, the risk of HBL for FAP patients is approximately 1000 times higher than that for the general population (12, 13). Therefore, genetic alteration in the APC gene is thought to be responsible for the tumorigenesis of HBL occurring in FAP. To determine whether inactivation of the APC gene plays a role in the development of sporadic HBL, 13 cases of infantile hepatic tumors were analyzed for genetic alterations in the APC gene.

MATERIALS AND METHODS

Samples. A total of 13 cases of infantile hepatic tumor were studied: 11 hepatoblastomas, 1 hepatocellular carcinoma, and undifferentiated hepatic sarcoma. None of the patients had a family history of FAP. Tumor tissues were obtained at the time of total resection prior to chemotherapy. A portion of each tumor was taken for histopathological examination, and the remainder was immediately frozen and stored at −80°C until DNA extraction. On the basis of histopathological examination, contamination by nontumor cells in each tumor was estimated to be <10%. In four cases, the corresponding normal tissues (peripheral blood or muscle) were also obtained. High-molecular-weigh DNA was extracted from both tumor and normal tissues with the standard SDS-protease K-phenol-chloroform method (14).

PCR-mediated RNase Protection Analysis. Since approximately 60–80% of the somatic mutation of colorectal tumors are reported to be clustered within a small region of exon 15, this region, designated as MCR, was first examined (11, 15). In addition, exons 3 and 4, where germinal mutations in attenuated FAP are known to cluster, were surveyed, because occurrence of one HBL in a patient in an attenuated FAP kindred had been reported previously (16, 17). Oligonucleotide primers for PCR were so designed that all of the regions of interest were covered (Table 1). PCR was performed with a thermal programmer (Perkin Elmer/Cetus Corp., Norwalk, CT) in volumes of 25 μl with 200 ng genomic DNA and 1.2 mm primers in 10% DMSO, 67 mm Tris-HCl (pH 8.8), 6.7 mm MgCl₂, 16.6 mm (NH₄)₂SO₄, 10 mm β-mercapto-ethanol, 6.7 mm EDTA, and 1.5 mm deoxynucleotide triphosphates containing 2.5 units Taq DNA polymerase (Boehringer Mannheim, Penzberg, Germany). PCR reactions consisted of 40 cycles of 40 s at 94°C, 2 min at 54°C (51°C for exons 3 and 4), and 2 min at 72°C.

The PCR products were screened for mutation by RNase protection analysis. The 32P-labeled RNA probes for each region representing the normal genomic sequences were prepared by using an RNA transcription kit (Stratagene, La Jolla, CA) and [α-32P]CTP (Amersham, Little Chalfont, England). One μl of amplified PCR products was hybridized with labeled RNA probes (1.5 × 10⁷ cpm) at 50°C for 2 h, after which the hybrids were digested with RNase A (22.5 mg/ml; Sigma, St. Louis, MO) at 37°C for 1 h. The RNase A was then inactivated by proteinase K (50 mg/ml) treatment and phenol-chloroform extraction. The RNA fragments were visualized by 8% polyacrylamide/8 urea denaturing gel electrophoresis followed by autoradiography. Two separate analyses were performed for each region, one with the sense and one with the antisense strand.

The variant PCR products were double digested with EcoRI and HindIII, and fractionated by electrophoresis through 2% agarose gel. The eluted DNA fragments were cloned in the EcoRI and HindIII sites of BluescriptII SK (Stratagene), and pools of at least 50 clones were sequenced together by means of the dideoxy chain termination method using a T7 sequencing kit (Pharmacia, Uppsala, Sweden). The primers used for sequencing were the T3 primer, 5'-ATTAAACCCCTACTAAG-3' (sense) and the T7 primer, 5'-AATACGACTCATAAG-3' (antisense).
LOH Analysis. We used two polymorphic probes on the long arm of chromosome 5: L5.71-3, a genomic fragment flanking the APC gene at 5q21, and SW41, a cDNA fragment of the 5' region of the APC gene (18, 19). For dosage determination, pEKZ19.3 (D9S17) on the long arm of chromosome 9 and C17 at 22q11 were used for the internal diploid standard (20, 21). These two marker loci were chosen because loss or gain have been rarely reported on these chromosomal arms in HBL.

Southern blot hybridization was carried out as reported previously (14). In brief, tumor/normal DNA pairs were completely digested with appropriate restriction enzymes, separated on agarose gel, and then blotted onto nylon membranes. DNA probes were radiolabeled with [α-32P]dCTP by means of the random primer method. Prehybridization and hybridization were performed overnight at 65°C in a hybridization solution containing 200 μg/ml salmon sperm DNA or sonicated human placental DNA. After hybridization, the blots were rinsed and then washed twice at 65°C with 0.1X SSC and 0.1% SDS for 15 min each, and exposed to Kodak XAR films at -80°C for 1 to 3 days.

Furthermore, Rsal polymorphism at exon 11 of the APC gene was also analyzed (22). PCR was carried out under the same conditions as described above, but with the following primers: G11A, 5'-TAGTATGTTGTTTTCCTCTT-3' and G11B, 5'-CTACACCTGACTTCTTAAAG-3'. The products were digested with Rsal, fractionated through 10% polyacrylamide gel, and visualized by ethidium bromide staining.

In Southern blot hybridization, the intensity of autoradiographic signals was measured densitometrically with a microcomputer imaging device (Imaging Research Inc., St. Catharines, Ontario, Canada). LOH was recorded when the ratio of alleles in the tumor tissue decreased to < 50% of the ratio in the corresponding normal tissue DNA. When the sample showed homozygosity at the locus, the intensity of the test locus was compared with that of the standard locus to determine the copy number of the locus. In the case of PCR-based LOH analysis, visual comparison was made between the signal intensity of normal tissues and tumor tissues. LOH was recorded only when complete loss or a remarkable decrease in density for one allele was observed.

RNA Analysis. Total RNA was isolated using the acid-guanidine-phenol-chloroform method with the aid of Isogen (Nippon Gene, Toyama, Japan) and isopropanol precipitation, cDNAs were dissolved in 10 μl dH2O.

The following primers were used for PCR amplifications of APC codons 125-220 (nucleotide 375–659); C33 on exon 3, 5'-TGGAGCGAGAAGAAGCTG-3'; and C34 on exon 5, 5'-ATTCTGCGCTATTTCTGCGCTG-3'. PCR reaction consisted of 40 cycles of 40 s at 94°C, 2 min at 54°C, and 2 min at 72°C. The PCR products were electrophoresed on a 10% polyacrylamide gel and visualized by ethidium bromide staining.

To confirm the specificity of the variant transcript, the PCR products were sequenced. Both normal and variant PCR products were fractionated by electrophoresis through a 2% agarose gel, and the eluted DNA fragments were cloned into the EcoRV sites of BluescriptII SK and sequenced as described above.

RESULTS

PCR mediated RNase protection analysis was performed on 13 infantile hepatic tumors to investigate sequence variations in the APC gene. One HBL sample (HBL6) showed variation in the region containing exon 4 of the APC gene (data not shown), and sequencing analysis of the variant PCR product revealed a G to T transversion at the splice site of the intron 3-exon 4 junction (Fig. 1). Since the mutation disrupted the acceptor site of the junction, it was assumed that this mutation resulted in expression of an aberrant APC transcript. Moreover, the normal sequence was not observed in the autoradiogram, indicating that the other allele had been lost in this tumor. Since sequence analysis of the DNA from normal muscle tissue of this patient showed both the mutant and normal alleles, the mutation was considered to have occurred in the germline. Since the parents of HBL6 did not carry the point mutation in their germline, the mutation identified in the patient was thought to have first occurred in the germinal cells of one of the parents.

To confirm the loss of the other allele, Southern blot analysis was performed for detection of LOH. Four cases, including HBL6, whose normal counterpart could be obtained were studied. When probed with L5.71-3, a genomic fragment flanking the APC gene, HBL6 showed LOH (Fig. 2), suggesting that the tumor had lost one allele of the APC gene. Although the autoradiographic signals of the control probes showed that loaded DNAs in the normal tissue and the tumor were approximately equal, the signal of the remaining allele was more intense in the tumor than the corresponding signal in the normal tissue. Because the patient showed constitutional homozygosity with the APC gene cDNA probe SW41, dosage analysis was carried out with two control probes as the internal diploid standards (Table 2). The comparison of the signal intensity of normal and tumor tissue
revealed that the tumor was diploid at the APC locus. This suggested, therefore, that the LOH was caused by an isodisomy resulting from chromosomal deletion and duplication, or from mitotic recombination. Accordingly, both of the APC genes in the HBL6 tumor were inactivated: one by germinally occurring point mutation and the other by somatic loss. The remaining three patients did not show LOH at any loci within or flanking the APC locus.

The point mutation detected in patient HBL6 was expected to eliminate the splice acceptor of this junction, resulting in expression of an aberrant APC transcript. Both tumor and normal muscle tissue were therefore examined for APC transcript by RT-PCR analysis. The result showed that the normal tissue of the patient yielded two types of PCR products in equal amounts. The larger product proved to correspond to the normal APC transcript, and the smaller was reduced in length by approximately 100 bp (Fig. 3). Thus, the germlinal mutation of this patient indeed resulted in expression of an aberrant APC transcript. On the other hand, tumor tissue of the patient yielded the aberrant and small amount of normal PCR products shown in Fig. 3. The results of LOH and sequence analysis suggested that the small amount of normal PCR product originated from the DNA of contaminated normal cells. It is, therefore, reasonable to consider that the tumor produced only the aberrant APC transcript due to somatic loss of the normal allele. Subsequent sequencing analysis of the aberrant PCR product showed conclusively that the mutation caused the abnormal splicing, resulting in the absence of exon 4 (Fig. 4). This abnormal splicing caused a frameshift with the termination signal appearing seven codons downstream of the junction.

![Fig. 2. Southern blot hybridization for examination of LOH at the APC flanking region.](image)

Each lane contains 5 mg MspI digested DNA from normal tissue (N) or tumor tissue (T) of HBL6. The probe is L5.71-3, which shows MspI polymorphism (arrows). LOH is observed in this case, as band A in Lane T is less intense than in Lane N. Furthermore, band B in Lane T is more intense than in Lane N (see "Results").

**Table 2 Dosage analysis at the APC locus of the patient**

<table>
<thead>
<tr>
<th></th>
<th>SW41 (5q)</th>
<th>pEKZ19.3 (9q)</th>
<th>C17 (22q)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Density</td>
<td>0.144</td>
<td>0.212</td>
<td>0.205</td>
</tr>
<tr>
<td>Tumor Density</td>
<td>0.091</td>
<td>0.135</td>
<td>0.132</td>
</tr>
<tr>
<td>Corrected density*</td>
<td>0.141</td>
<td>0.210</td>
<td>0.205</td>
</tr>
<tr>
<td>Copy number*</td>
<td>1.96</td>
<td>1.98</td>
<td>2.00</td>
</tr>
</tbody>
</table>

*Corrected density in the tumor tissue was calculated by the ratio of C17 density of the normal and tumor tissue in order to obtain correction of variations in DNA loading. The copy number of each locus in the tumor tissue was estimated by establishing the copy number in the normal tissue as 2.00.

**DISCUSSION**

To date, the mechanism of tumorigenesis in most childhood tumors has to some extent been genetically explained. However, there has been no consistent evidence of involvement of specific genes in the tumorigenesis of HBL. FAP patients often suffer from extracolorectal malignant tumors such as gastric and duodenal cancer as well as HBL. The risk of gastric cancer, which is the most common malignant tumor worldwide, is at least 10 times higher for FAP patients than for the general population. Gastric cancer frequently shows LOH on chromosome 5, and several sporadic gastric tumors have been reported with a somatic mutation in the APC gene (23, 24). The inactivation of the APC gene is, therefore, considered to play an important role during carcinogenesis of at least some gastric cancers. The risk of HBL for FAP patients is reported to be approximately 1000 times higher than that for the general population. The much higher frequency of HBL than of gastric cancer in FAP cases strongly suggests a close relationship between the tumorigenesis of HBL and the inactivation of the APC gene.

In this study, genetic alterations of the APC gene were examined in 13 cases of infantile hepatic tumor, resulting in identification of one HBL with genetic alterations on both alleles of the APC gene. The tumor had lost one of the APC alleles, and a point mutation was detected at an intron-exon junction of the remaining allele. The mutation was thought to have affected normal splicing, and, indeed, RNA analysis detected the aberrant APC transcript in which exon 4 was completely absent. Because elimination of exon 4 caused a frameshift resulting in termination at seven codons downstream of the junction, this aberrant transcript supposedly produced a truncated APC protein. One other report of a point mutation at this junction in a sporadic colorectal adenoma suggests that this mutation definitely causes inactivation of the APC gene (25). It is, therefore, thought that no normal APC protein could be expressed in this tumor due to biallelic inactivation of the APC gene. At present, inactivation of both alleles of the APC gene is considered to be essential for development of colorectal tumor in FAP, even in sporadic cases (10, 11). Moreover, high frequency of somatic mutation in the APC gene has been reported in gastroduodenal or desmoid tumors occurring in FAP patients (26–28). The biallelic inactivation of the APC gene observed in HBL6 thus conceivably represents a specific change for tumorigenesis.

Although the mutation identified in the HBL6 tumor was confirmed...
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Fig. 4. DNA sequences of the RT-PCR product from HBL6. A, DNA sequence of the RT-PCR product from the normal (left) and the aberrant (right) APC transcripts. In the aberrant transcript, exon 4 is eliminated by abnormal alternative splicing. B, schema of the abnormal splicing of the aberrant transcript. This splicing results in a frame-shift with the termination signal appearing seven codons downstream of the junction.

A germinal mutation, no somatic mutation of the APC gene was identified in the remaining 12 tumors. One possible explanation is that inactivation of the APC gene might play a causative role only in HBL occurring in FAP patients, while sporadic HBL is assumed to develop through another pathway without genetic alterations of the APC gene. Of course, because of the limited number of cases and the limited extent of the region examined in the present study, there still remains the possibility that inactivation of the APC gene is closely related to tumorigenesis of sporadic HBL as well. Because the size of the APC gene is approximately 9 kb, the region examined in this study covered only one sixth of the entire APC gene. Although karyotypic analysis of HBL has not shown any consistent alteration of 5q, LOH due to chromosomal loss and duplication or to mitotic recombination cannot be detected by karyotypic examination. LOH at 11p15, which is frequently observed in HBL, has been reported as being due to isodisomy resulting from mitotic recombination (5). Accordingly, LOH on 5q, as well as at 11p15, might occur more frequently than is indicated by karyotypic examination. A more comprehensive study including a larger number of tumors and examination of the entire region of the gene should clarify the exact role of the APC gene in the tumorigenesis of sporadic HBL.

Although the risk of HBL is higher for FAP patients than for the general population, not all of the former have become HBL patients. Interestingly, one FAP family was reported to have two members who suffered from HBL (13). There is also a report of identical twins, both of whom suffered from HBL (29). Therefore, it is reasonable to assume that the occurrence of HBL in FAP is associated with germinal mutation at a specific region of the APC gene. In fact, genotype-phenotype correlation has been established in FAP according to the location of the germinal mutation. Such a mutation of the 5' region of the gene has been responsible for one characteristic type of FAP, the so-called attenuated type, which is characterized by development of a relatively small number of polyps (17). One report mentions an infant in one kindred of attenuated FAP who died from HBL (16). The germinal mutation identified in our case HBL6 was located at exon 4, which is located in the 5' region of the APC gene. The germinal mutation of this region of the gene may thus be responsible for development of HBL in FAP kindreds. A further search for the location of the germinal mutation of the gene in FAP patients who develop HBL should clarify this point.

In this study, detailed genetic analysis was performed of the APC gene in infantile hepatic tumors, resulting in identification of somatic inactivation of the APC gene in a patient with germinal point mutation of the APC gene. This finding provides an important clue for understanding the molecular genetic mechanism of HBL tumorigenesis and also confirms the association and risk of HBL development in FAP patients.

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