Biallelic Inac tion of the *APC* Gene Is Associated with Hepatoc lar Carcinoma in Familial Adenomatous lyposis Coli

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BACKGROUND. Certain primary hepatic tumors have been associated with familial adenomatous polyposis (FAP), a condition caused by germline mutations of the adenomatous polyposis coli (*APC*) gene. However, a genetic association between FAP and hepatocellular carcinoma (HCC) has not been shown. This study tested the hypothesis that biallelic inactivation of the *APC* gene contributed to the development of HCC in a patient with FAP and a known germline mutation of the *APC* gene at codon 208, but no other risk factors for HCC.

METHODS. Total RNA and genomic DNA were isolated from the tumor, and in vitro synthesized protein assay and DNA sequencing analysis were used to screen for a somatic mutation in the *APC* gene.

RESULTS. A somatic one–base pair deletion at codon 568 was identified in the wild-type allele of the *APC* gene.

CONCLUSIONS. To the authors' knowledge, this study provides the first evidence that biallelic inactivation of the *APC* gene may contribute to the development of HCC in patients with FAP. ***Cancer* 2001;92:332–9.**

KEYWORDS: familial adenomatous polyposis, Gardner syndrome, adenomatous polyposis coli (APC), loss of heterozygosity, hepatocellular carcinoma, in vitro synthesized protein assay.

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease characterized by the presence of numerous adenomatous polyps throughout the colon and rectum. Polyps are usually clinically evident by the teen years. Progression of one or more colonic adenomas often leads to colorectal carcinoma in patients with FAP.[1] Germline mutations in the adenomatous polyposis coli (*APC*) gene have been found in many patients with FAP.[2–5] In addition, somatic mutations in the *APC* gene have been found in colorectal tumors in patients with and without FAP.[6–8] Almost all identified *APC* mutations result in truncation of the *APC* protein. The APC protein has been shown to function as a tumor suppressor by regulating the transcriptional activity of β-catenin. APC protein promotes the degradation of free β-catenin, a process that also involves axin and glycogen synthase kinase 3β.[9–12] Colorectal tumors that do not have *APC* mutations often express β-catenin mutants that are resistant to regulation by *APC*.[11,13]

Patients with FAP can have extracolonic manifestations of their disease.[1] These include tumors of the upper gastrointestinal tract, such as adenomas, microcarcinoids, and hyperplastic and lymphoid polyps; osteomas of the mandible, skull, and long bones; and a variety

of soft tissue lesions, including fibromas, lipomas, and desmoid tumors. Familial adenomatous polyposis that is associated with cutaneous and soft tissue lesions is referred to as Gardner syndrome;[14] patients with this syndrome are clinically but not genetically different from others with FAP. Since the 1950s, a variety of hepatic neoplasms have been described in patients with FAP (see Table 1). Recently, biallelic inactivation of the *APC* gene was described in one hepatocellular adenoma[15] and one hepatoblastoma.[16] In the current study, we investigated the role of *APC* mutation in the development of hepatocellular carcinoma (HCC) in a patient with FAP. The patient had clinical manifestations of Gardner syndrome and a known germline mutation of the *APC* gene. To our knowledge, this is the first report of a molecular analysis of the *APC* gene in a case of HCC that developed in a patient with FAP.

MATERIALS AND METHODS

Clinical Summary

The family history of this patient has been reported previously.[17] This patient is the son of the proband described in the earlier report. The patient had a germline *APC* mutation that changed codon 208 from coding for glutamine (CAG) to coding for a stop codon (TAG; family MDA033 in Su et al.[18]). The patient received a diagnosis of FAP in 1986 at age 15 and underwent proctocolectomy in 1994. In 1996, he developed unresectable desmoid tumors in the periappendiceal region and retroperitoneum that caused right-sided hydronephrosis and necessitated ureteral stenting. The patient was treated initially with tamoxifen and later with methotrexate and vinblastine; the total duration of chemotherapy was less than 12 months. The growth of the desmoid tumors stabilized, and the chemotherapy was discontinued. The patient underwent routine computed tomography scanning every 6 months.

In July 1999, a new hepatic mass measuring 5.8 $\times$ 4.4 cm was discovered on imaging, and the patient was referred for consideration of hepatic resection. Staging evaluation revealed that the lesion was resectable and that the patient had no extrahepatic disease except for the known desmoids. The patient had no known risk factors for HCC. The patient denied any alcohol abuse, hepatitis B and C serology were negative, and carcinoembryonic antigen and α-fetoprotein were within normal range. Endoscopy revealed tubular adenomas in the duodenum but no other gastrointestinal manifestations of FAP. The patient underwent an uncomplicated right hepatic trisegmentectomy in February 2000. Pathologic examination of the surgical specimen revealed a single, well differentiated, encapsulated HCC without vascular invasion (Fig. 1A). There was no underlying hepatitis, fibrosis, or cirrhosis in the adjacent liver parenchyma (Fig. 1B). At the time of this report, the patient had no evidence of recurrent disease and had fully recovered from the hepatic resection.

Molecular Analysis of the *APC* Gene

The patient gave informed consent for genetic analysis of his tumor. The institutional review board approved all sample collection and tumor analysis. The tumor tissue was snap-frozen at the time of resection. Total RNA was isolated from the tumor tissue using Trizol reagent (Life Technologies, Gaithersburg, MD) and used for the synthesis of the first strand cDNA by using the SuperScript Preamplification system (Life Technologies) according to the manufacturer's instructions. Genomic DNA was isolated from the tumor tissue by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Total RNA and genomic DNA isolated from blood of an unrelated FAP patient were used as control samples in this molecular analysis.

Various methods that are commonly used for DNA mutation analysis have been used to detect *APC* mutations. These methods include single-strand conformation polymorphism (SSCP),[2] denature gradient gel electrophoresis (DGGE),[19] and RNase protection.[5] These are effective methods to analyze small DNA fragments, but inefficient for analysis of *APC* because of the large coding region of this gene. After some initial *APC* mutation analyses, it became obvious that most if not all *APC* mutations resulted in truncation of the APC protein (nonsense mutations, reading frameshift deletions or insertions). An efficient method for analysis of the entire coding region of *APC* for truncating mutations was developed independently by two laboratories and has been called the in vitro synthesized protein assay (IVSP) or protein truncation test (PTT).[20–22] In addition to being a rapid method, IVSP and PTT can detect genomic rearrangements that delete exons or that result in splicing defects because they can analyze cDNA instead of genomic DNA.[18] These genomic rearrangements cannot be detected using methods such as SSCP and DGGE that analyze each exon individually. We used the IVSP to identify potential somatic *APC* mutation in this case as we have described previously.[18] The coding region of *APC* was amplified in three overlapping segments (segment 1, codons 1 to 811; segment 2, codons 680 to 1692; and segment 3, codons 1547 to 2843). All polymerase chain reactions (PCRs) were performed by using the Expand Long Template PCR System (Boehringer Mannheim, Indianapolis, IN) in the final volume of 25 μL as fol-

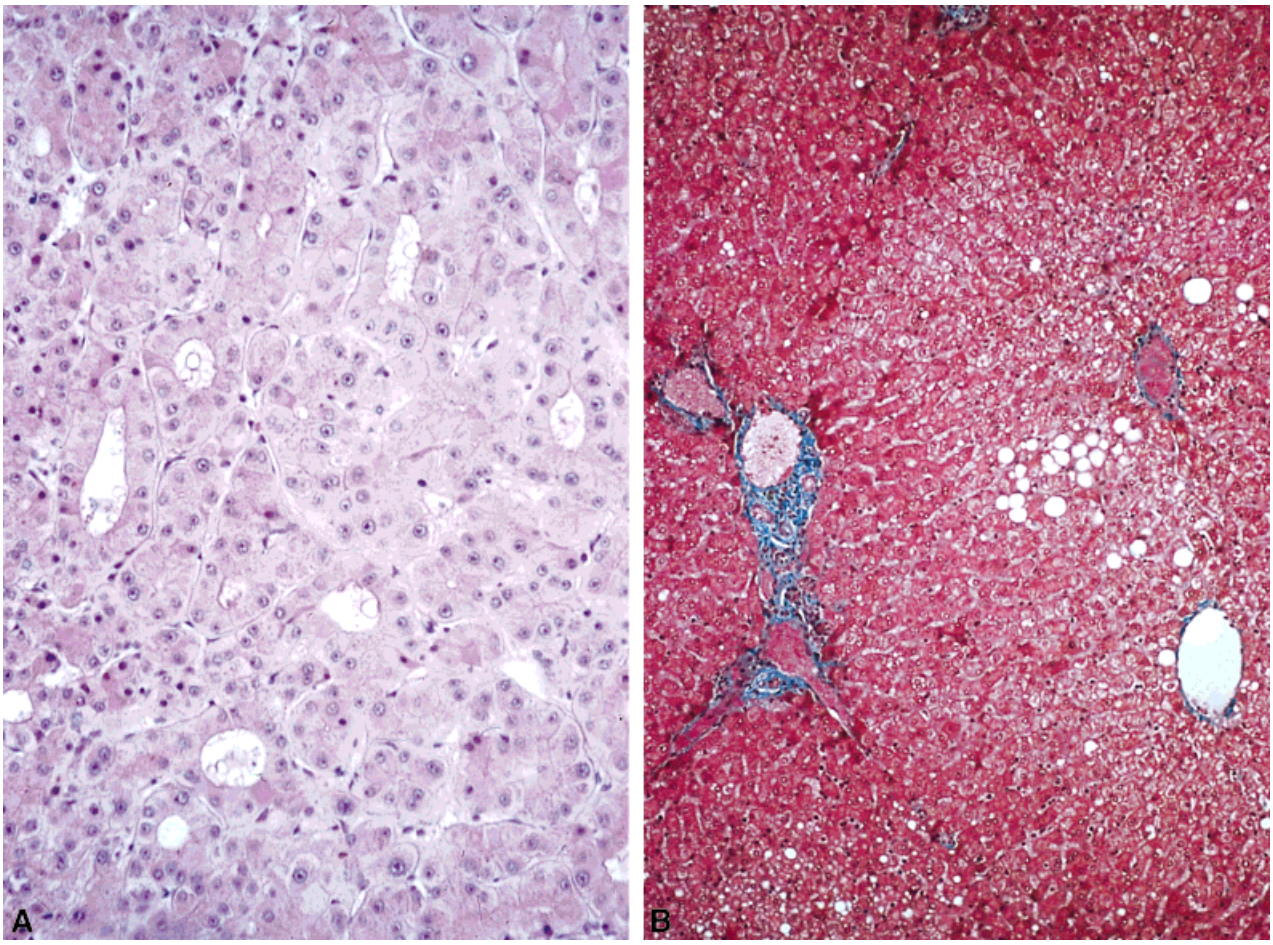

FIGURE 1. Hepatocellular carcinoma and surrounding normal hepatic parenchyma. (A) Hepatocellular carcinoma with trabecular and pseudoglandular pattern composed of large, polygonal cells with abundant cytoplasm and nuclear pleomorphism (H & E stain original magnification ×160). (B) Adjoining nonneoplastic liver showing focal macrovesicular steatosis but no fibrosis or cirrhosis (Masson's Trichrome stain, original magnification ×16).

lows: 1 cycle at 92 °C for 2 minutes; 35 cycles each at 92 °C for 15 seconds, 55 °C for 1 minute, and 68 °C for 2.5 minutes; and 1 cycle at 68 °C for 5 minutes. Segment 1 was amplified in two steps using cDNA as the template. The first-step PCR used the upstream primer 5′-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGCTGCAGCTTCATATGATC-3′ and the downstream primer 5′-ATAAGGCATAGAACATGTCC-3′. One microliter of the reaction product was used as the template in the second-step PCR using the same upstream primer and the downstream primer 5′-CTGACCTATTATCATCATGTCG-3′. Segments 2 and 3 were amplified using genomic DNA as the template. The upstream primer 5′-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGATGCATGTGGAACTTTGTGG-3′ and the downstream primer 5′-TTCTGTAGGAATGGTATCTCG-3′ were used to amplify segment 2. The upstream primer 5′-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGAAAACCAAGAGAAAGAGGC-3′ and the downstream primer 5′-GAGTGGATCCCAAAATAAGACC-3′ were used for the amplification of segment 3. The coupled in vitro transcription and translation reactions were performed in the presence of ^{35}S-methionine by using the T7 TNT System (Promega, Madison, WI). The products were resolved on 10–20%% gradient sodium dodecyl sulfate–polyacrylamide gels (Bio-Rad, Hercules, CA) and detected by autoradiography using Kodak BioMax film (Rochester, NY).

To determine the allele that carried the somatic mutation, we cloned the PCR product of segment 1 by using the Original TA Cloning Kit (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated from six clones. The regions containing the germline mutation (codon 208) and the somatic mutation (codon 568) of each clone were sequenced.

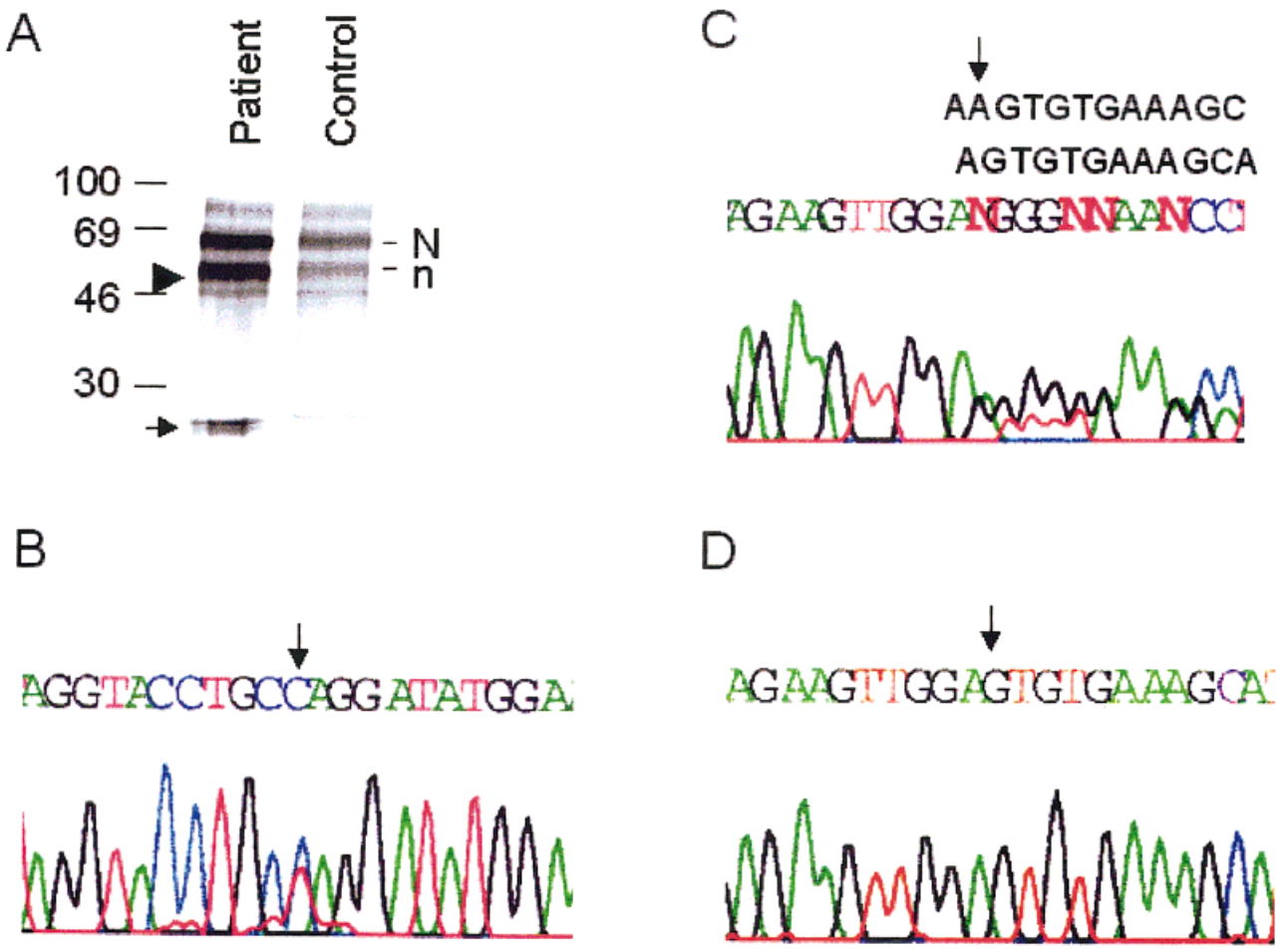

FIGURE 2. Mutation analysis of *APC*. (A) Identification of *APC* mutation by IVSP. Only the result of segment 1 is shown. N and n indicate the slower-migrating and faster-migrating normal products, respectively. The small arrow indicates the mutant product resulting from the germline mutation. The large arrowhead indicates the increased intensity of the faster-migrating product. (B) Germline *APC* mutation. The arrow indicates the single nucleotide alteration that changed codon 208 from CAG (coding for glutamine) to TAG (stop codon). (C) Somatic *APC* mutation. Parts of the wild-type (top) and the mutant (bottom) sequences are shown above the sequencing chromatogram. The arrow indicates the single base pair deletion. (D) Sequence of the somatic mutant *APC* allele. The arrow points to the deletion (AA to A).

RESULTS

Mutation analysis of the *APC* gene in the HCC from this patient confirmed the presence of the germline mutation at codon 208 (Figs. 2A,B). No other mutant IVSP product was apparent (Fig. 2 and data not shown). However, in the tumor, the fast-migrating IVSP product of segment 1 was as abundant as the slower-migrating product (Fig. 2A). In contrast, the control sample had less fast-migrating IVSP product of segment 1 than the slower-migrating product. Our previous study[18] has shown that this finding indicates a somatic mutation in segment 1 near exon 14. DNA sequencing of segment 1 identified a one–base pair deletion at codon 568 (AGT to GT; Fig. 2C). This mutation was confirmed by sequencing *APC* exon 13 amplified using genomic DNA isolated from the tumor (data not shown). This mutation was not found in the same *APC* exon amplified using genomic DNA isolated from adjacent normal liver (data not shown). To determine whether the somatic mutation was present on the germline wild-type allele of *APC*, the product of a second reverse transcription–PCR of segment 1 was cloned. Plasmid DNA was isolated from several individual clones and sequenced. The result showed that the somatic mutation and the germline mutation were not on the same clone. Thus, the somatic mutation was on the germline wild-type allele (Fig. 2D and data not shown).

DISCUSSION

We found biallelic inactivation of the *APC* gene in a HCC from a patient with Gardner syndrome. The pa-

tient had a germline *APC* mutation at codon 208, and the tumor contained an additional somatic *APC* mutation at codon 568 on the wild-type allele. The somatic *APC* mutation was not found in the surrounding normal liver, and there were no clinical or pathologic signs suggesting other risk factor for HCC. These findings provide a genetic basis for the association between FAP and HCC and suggest a role for APC/β-catenin pathway in the hepatic tumorigenesis of patients with FAP.

β-Catenin performs at least two key functions in cells.[10] First, it plays a role in cell–cell adhesion. Second, β-catenin, through interaction with Tcf/Lef, is a signaling molecule involved in transcription and activation of target genes. The APC protein downregulates β-catenin by promoting the degradation of free β-catenin.[23] As previously shown in colon carcinogenesis,[9,11,13] disruption of normal APC/β-catenin functions by mutation of the *APC* or β-catenin genes results in β-catenin accumulation in cells, leading to upregulation of β-catenin target genes.

Patients with FAP have been shown to be at increased risk for developing hepatic tumors. Hepatoblastomas occur in young patients with FAP at least 100 times more often than in the general population.[24–26] Biallelic mutation of the *APC* gene in a hepatoblastoma in a patient with a germline mutation of *APC*[16] and the existence of β-catenin mutations in sporadic hepatoblastoma have been reported.[27–29] Sporadic HCCs also have been found to contain β-catenin mutation and accumulation.[8,30–33] Although studies have examined the loss of heterozygosity at the *APC* locus in HCC,[34] our report is the first to our knowledge to characterize a biallelic inactivation of the *APC* gene in a HCC.

Note that our patient received a short course of tamoxifen, a putative hepatocellular carcinogen, before the development of HCC. Tamoxifen-induced DNA adduct formation in hepatocytes has been proposed as a causative factor in the development of HCC in selected rat strains, suggesting that differing genetic susceptibility plays a role. Although two clinical reports have described the association between the development of HCC and tamoxifen in humans,[35,36] humans are thought to be much less susceptible to tamoxifen-induced DNA adduct formation than rats.[37] Although tamoxifen currently is not considered to be a primary hepatic carcinogen in humans, the possibility of a role for tamoxifen in hepatocellular carcinogenesis among genetically susceptible patients remains.

This patient also received a short course of methotrexate (cumulative dose <1 g over 24 weeks). Long courses of methotrexate for treatment of leukemia or psoriasis have been associated with hepatic fibrosis, and in rare cases hepatoma in a setting of "methotrexate induced" hepatic fibrosis.[38,39] These patients typically are treated for more than 5 years, with cumulative methotrexate doses of 2.5 g in an 11-year-old girl[38] and approximately 4 g in an adult.[39] Certainly, the association between methotrexate and hepatic fibrosis is accepted, but the link to HCC is difficult to clarify given that most cases occur as a second malignant neoplasm in patients with existing cancers (particularly acute leukemias, with recognized genetic defects).[40] Even in patients with prior HCC, risk for recurrence or second primary HCC relates to existence of underlying fibrosis,[41] which was absent in this patient (Fig. 1B). Hutter et al.[42] examined pathologic specimens from livers of leukemic children before and after the era of chemotherapy.[42] They found that only 4% of leukemic children who did not receive chemotherapy had moderate hepatic fibrosis, and none had severe fibrosis. Thirty-one percent of those who received antifolate/steroid therapy and 42% of those who received antipurine/anti-folate/steroid therapy had moderate or severe hepatic fibrosis. None of the 273 patients was found to have HCC. Still, the survival in chemotherapy group was more than double the survival in the no chemotherapy group further clouding the analysis, and no such study could be repeated given the current standard of care for leukemia.[42] Given the tenuous relation between drug-induced chronic liver disease and hepatocellular carcinoma and the absence of definite fibrotic changes in the underlying liver, methotrexate is unlikely to have contributed to hepatocellular carcinoma in the current study.

Many investigators have attempted to identify an association between the location of germline *APC* mutations and the occurrence of extracolonic manifestations of FAP. Desmoid tumors have been shown to occur more frequently (though not always) when germline mutations occur on the 3′ half of the *APC* gene.[43–45] The patient described in this report is one of the exceptions; he developed desmoid tumors, but his germline *APC* mutation was at the 5′ end of *APC* (at codon 208).

We reviewed all reported cases of hepatocellular neoplasms in patients with FAP (Table 1). Germline *APC* mutations have been identified in only a few of these patients. There is no apparent correlation between the location of germline *APC* mutations and the occurrence of these tumors. Giardiello and colleagues described eight cases of hepatoblastoma in patients with FAP and Gardner syndrome and found different germline *APC* mutations between codons 141 and 1230.[46] Bala and colleagues described a hepatocellular adenoma in a patient with a germline *APC* mutation at

TABLE 1
Reported Cases of Primary Hepatic Tumors in Association with FAP Syndromes and APC Mutations

Hepatic tumor	First author (year)	No. of cases	Polyposis syndrome	Germline mutation (codon number)	Somatic mutation (codon number)
Adenoma	Bala et al. (1997)[15]	1	FAP	1451	LOH
Hepatoblastoma	McNab et al. (1952)[50]	1	FAP	—	—
	Kingston et al. (1982)[24]	3	FAP[a]	—	—
	Kingston et al. (1983)[51]	5	FAP	—	—
	Weinberg and Finegold (1983)[52]	1	FAP	—	—
	Kasukawa et al. (1985)[53]	1	FAP	—	—
	Li et al. (1987)[54]	4	FAP	—	—
	Garber et al. (1988)[55]	11	FAP[a]	—	—
	LeSher et al. (1989)[56]	1	FAP	—	—
	Phillips et al. (1989)[57]	1	FAP	—	—
	Toyama and Wagner (1990)[58]	1	FAP	—	—
	Riikonen et al. (1990)[59]	2	Presumed FAP	—	—
	Bernstein et al. (1992)[60]	2	FAP	—	—
	Hughes and Michels (1992)[26]	2	FAP	—	—
	Kurahashi et al. (1995)[16]	1	—	Intron 3–exon 4 junction	LOH
	Giardiello et al. (1996)[46]	8	FAP[a]	141, 215, 302, 541, 1061, 1189, 1230, 1230	—
	Cetta et al. (1997)[61]	1	FAP	1061	—
Hepatocellular carcinoma	Veale (1965)[62]	1	FAP	—	—
	Weinberger et al. (1981)[63]	1	Gardner	—	—
	Zeze et al. (1983)[64]	1	FAP	—	—
	Laferla et al. (1988)[65]	1	FAP	—	—
	Van Steenbergen et al. (1989)[66]	1	FAP	—	—
	Spigelman et al. (1991)[67]	1	FAP	—	—
	Gruner et al. (1998)[47]	2	Gardner	Between 1099 and 1693	—
			FAP	—	—
	Current case	1	Gardner	208	568

FAP: familial adenomatous polyposis; APC: adenomatous polyposis coli; LOH: loss of heterozygosity.
[a] FAP and Gardner syndrome not distinguished in these reports.

codon 1451.[15] Gruner and colleagues described a fibrolamellar variant of HCC in a patient with a germline mutation in segment 3 of the *APC* gene, which contains codons 1099 to 1693.[47] The patient described in our report had a germline mutation at *APC* codon 208. Further strengthening the hypothesis that the patient in the current report was genetically susceptible to mutation is the negative study of Chen et al.[48] These investigators examined hepatocellular carcinomas in 46 patients of whom 24 had cirrhosis and 43 had a history of hepatitis. They could show no loss of heterozygosity of the *APC* or mutated in colorectal carcinoma gene loci by fragment length polymorphism analysis or PCR and concluded that such a gene defect in the genesis of HCC is likely very rare. Identification of a specific codon or set of codons that play a role in HCC in FAP will be difficult given the low incidence of the disease.

In summary, this study indicates that biallelic *APC* inactivation may promote HCC or may lead to a genetic instability in hepatocytes that, when combined with other genetic or extraneous factors, leads to HCC. Further study of germline and somatic *APC* mutations and β-catenin pathway in FAP may help to unfold the events leading to HCC in the subset of patients who develop HCC in the absence of chronic liver disease.[49]

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