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Aberrations of the K-*ras*, *p53*, and *APC* Genes in Extrahepatic Bile Duct Cancer

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Background and Objectives: The genetic alterations involved in extrahepatic bile duct (EHBD) cancer are poorly understood. Our aim was to identify aberrations of the K-*ras*, *p53*, and *APC* genes in EHBD cancer.
Methods: We investigated aberrations of these genes in 52 EHBD cancers using polymerase chain reaction (PCR) single-strand conformation polymorphism analysis, followed by direct sequence determination and a PCR restriction fragment length polymorphism assay.
Results: The K-*ras*, *p53*, and *APC* genes were mutated in 9.6%, 32.7%, and 0% of EHBD cancers, respectively. Loss of heterozygosity at the *p53* and *APC* gene loci was identified in 15.6% and 38.5% of EHBD cancers, respectively.
Conclusions: Our results suggest that an unknown suppressor gene on 5q other than the *APC* gene may be responsible for EHBD cancer.
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KEY WORDS: **carcinoma of the extrahepatic bile duct; K-*ras; p53; APC***

INTRODUCTION

Carcinogenesis is a multistep process characterized by several genetic alterations, including mutational activation of proto-oncogenes and inactivation of tumor-suppressor genes by mutations and loss of the normal allele [1]. The accumulation of genetic alterations within *APC*, K-*ras*, and *p53* is considered crucial to the course of colorectal tumorigenesis [1]. Genetic alterations in colorectal tumors could similarly combine during the course of carcinogenesis in other epithelial tumors. However, whether or not these genetic alterations are related to the pathogenesis of cancer of the extrahepatic bile duct (EHBD) remains unclear. The aim of this study was to characterize the mutational features of the K-*ras*, *p53*, and *APC* genes in EHBD cancer. Mutations of the 3 genes were examined by polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing. Loss of heterozygosity (LOH) was evaluated by PCR-restriction fragment length polymorphism (PCR-RFLP) assay using polymorphic sites within the *p53* and *APC* genes. In addition, we assessed the relationship between these genetic alterations and clinicopathological features.

MATERIALS AND METHODS

Patients

Tissue samples were obtained from 52 patients with sporadic EHBD adenocarcinoma excluding cancer of the ampulla of Vater (37 men and 15 women, mean age 66.8 years). Some had undergone pancreatoduodenectomy (44 cases) and choledochectomy (8 cases) with extensive lymph node dissection at the Department of Surgery I, Iwate Medical University Hospital, from July 1990 to

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TABLE I. Frequencies of Alterations of K-ras, p53, and APC Genes

Mutations			LOH	
K-ras (exon 1)	p53 (exon 5–8)	APC (MCR)[a]	p53	APC
5/52[b] (9.6%)	17/52 (32.7%)	0	5/32[c] (15.6%)	10/26 (38.5%)

[a]MCR, mutation cluster region, [b]positive/total samples, [c]positive/informative samples.

TABLE II. Direct Sequences of K-ras and p53 Genes

Cases	Genes	exon	codon	Nucleotide changes	Amino acid changes
2	K-ras	1	12	GGT to TGT	Gly to Cys
8	K-ras	1	12	GGT to TGT	Gly to Cys
13	K-ras	1	12	GGT to TGT	Gly to Cys
51	K-ras	1	12	GGT to GAT	Gly to Asp
63	K-ras	1	12	GGT to GTT	Gly to Val
1	p53	5	—	not determined	—
6	p53	5	175	CGC to CAC	Arg to His
21	p53	5	147	GTT to GGT	Val to Gly
22	p53	5	157	GTC to GAC	Val to Asp
69	p53	5	158	CGC to CAC	Arg to Tyr
56	p53	6	212	CGA to TGA	Arg to Stop
3	p53	7	242	TGC to TGG	Cys to Trp
7	p53	7	248	CGG to TGG	Arg to Trp
12	p53	7	229	TGT to TGA	Cys to Stop
46	p53	7	239	AAC to GAC	Asn to Asp
11	p53	8	—	not determined	—
27	p53	8	—	not determined	—
53	p53	8	273	CGT to AGT	Arg to Ser
54	p53	8	273	CGT to CAT	Arg to His
57	p53	8	273	CGT to CAT	Arg to His
63	p53	8	265	CTG to ATG	Leu to Met
65	p53	8	265	CTG to ATG	Leu to Met

December 1996. All surgical operations were performed with curative resection. The pathological stage of the disease was based on the TNM classification of malignant tumours [2]. Early cancer, classified according to the Japanese Society of Biliary Surgery [3], corresponded to Tis and T1 of the TNM classification of EHBD cancer.

DNA Extraction

Tumor and matching normal tissues were immediately frozen after surgery and verified by hematoxylin staining. High-molecular-weight DNA was isolated by lysis in sodium dodecyl sulfate and by proteinase K digestion, then extracted using phenol chloroform. All tumor specimens consisted of at least 60% neoplastic cellularity.

PCR

The published sequences of the oligonucleotide primers were used for PCR-SSCP [4–6] and PCR-RFLP [7–9]. Extracted genomic DNA was amplified in PCR mixtures according to the described protocol with a slight modification [10]. PCR was started with a denaturation step of 5 min at 94°C, followed by 35 cycles each consisting of denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec (*p53* exons 5, 6, and 7 for SSCP) or at 55°C for 30 sec (*p53* exon 8, K-*ras* exon 1, and *APC* exon 15 for SSCP and *p53* intron 1, exon 4 and *APC* exon 11 for RFLP), and extension at 72°C for 1 min, with a final extension of 5 min at 72°C using the PCR System 9600 (Perkin-Elmer Cetus, Norwalk, CT). Genomic DNA samples derived from colon cancers were the positive control for these analyses.

Nonradioisotopic PCR-SSCP Analysis

PCR products were diluted with formamide dye [10], denatured at 94°C for 3 min in a heat block then kept on ice. Samples were loaded onto a 12% polyacrylamide gel for the K-*ras* gene or a 7.5% polyacrylamide gel including glycerol for the *p53* and *APC* genes. Electrophoresis proceeded at 300 V for 2–3 hr for the *p53* gene, 180 V for 10–14.5 hr for the *APC* gene, and 400 V for 3.5 hr for the K-*ras* gene at 23°C using a temperature controller (Resolmax; ATTO, Tokyo, Japan). The gel was stained with silver [11], and mutations were identified by the presence of extra bands that separated from the normal control band.

Direct DNA Sequencing

Direct sequencing by dideoxy chain termination was performed when SSCP analysis detected a shifted band. Amplified products were purified using SUPREC™-01 (Takara Shuzo, Kyoto, Japan), then precipitated with ethanol. Purified DNA was sequenced as follows. The initial cycle of 3 min at 96°C was followed by 25 cycles each consisting of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and extension at 60°C for 4 min, using a thermal cycler (PCR system 9600) and the ABI PRISM™ cycle sequencing kit (Perkin-Elmer Cetus). Electrophoresis and analysis were performed using a DNA sequencer (model 373A; Perkin-Elmer Cetus).

PCR-RFLP assay

PCR products were digested for 3 hr at 37°C with BglII (Takara Shuzo) for *p53* intron 1, AccII (Takara Shuzo) for *p53* exon 4, and RsaI (Takara Shuzo) for *APC* exon 11. Digests were resolved by electrophoresis on a 3% agarose gel for *p53* intron 1 at 50 V for 1 hr and an 8% polyacrylamide gel for *p53* exon 4 and *APC* at 120 V for 1 hr, then stained with ethidium bromide and photographed under ultraviolet light. LOH was defined as a visible change in the allele: allele ratio in the tumor DNA relative to the ratio in the corresponding normal DNA

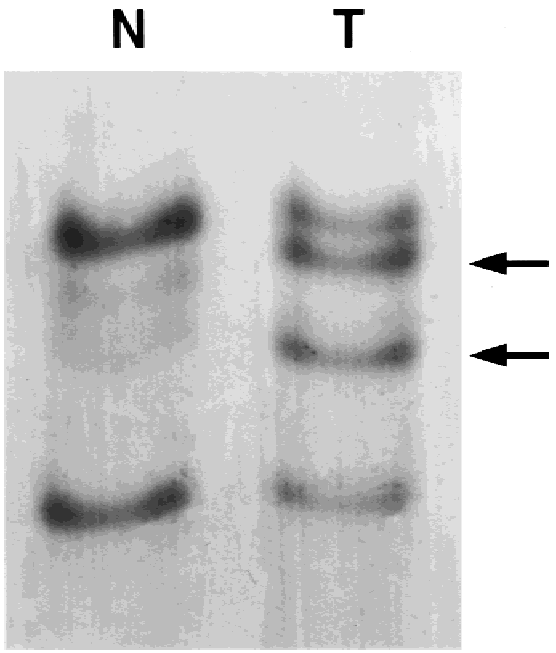

Fig. 1. Polymerase chain reaction single-strand conformation polymorphism analysis for detecting mutations in exon 1 of the K-*ras* gene. Mobility shift bands are indicated by arrows. N, normal DNA; T, tumor DNA.

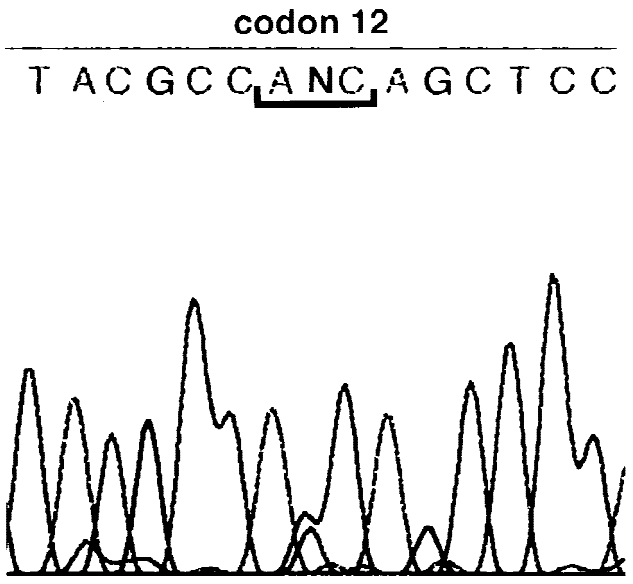

Fig. 2. Sequence histogram (antisense) of exon 1 of the K-*ras* gene. Point mutation is located at the second letter of codon 12 (underlined). The nucleotide change is a GGT to GAT transition (sense sequence).

[12]. LOH positivity for the *p53* gene was defined as LOH for at least 1 polymorphic site.

Statistical Analysis

Statistical differences between several variables and gene alterations were analyzed using the χ^2 test or the Fisher exact method. Overall survival curves were estimated by the Kaplan-Meier method, and statistical significance was analyzed by log-rank statistics. Differences were considered statistically significant when the probability value was below 0.05.

RESULTS

Representative results for K-*ras* gene mutations are shown in Figures 1 and 2. The K-*ras* gene was mutated in 5 of the 52 EHBD cancers (9.6%). All of the point mutations were demonstrated in codon 12. The most frequent mutations were transversions from GGT to TGT (3 of the 5 mutated cases, 60%).

Mobility shifts of the *p53* gene were detected in 17 of the 52 EHBD cancers (32.7%): 5 (29.4%) in exon 5, 1 (5.9%) in exon 6, 4 (23.5%) in exon 7, and 7 (41.2%) in exon 8 by PCR-SSCP analysis. Direct sequencing revealed 14 point mutations; 2 were nonsense mutations resulting in a truncated p53 protein, and 12 were missense mutations (6 transitions and 6 transversions). Nucleotide changes could not be determined in the 3 remaining shifted bands. The results of PCR-RFLP assay for the *p53* gene locus are shown in Figure 3. The frequency of allelic loss on the *p53* gene was 15.6% (5 of 32 informative samples).

APC gene mutation was not found in EHBD cancer by PCR-SSCP analysis, whereas an LOH at the *APC* gene locus was identified in 10 (38.5%) of 26 informative samples.

The difference in the K-*ras* mutational rate between early (pTis and pT1, n = 8) and advanced (pT2 and pT3, n = 44) cancers was significant ($P = 0.02$). The others were equally distributed between early and advanced cancers and between cancers with and without lymph node metastasis.

Survival rates of the groups with (n = 5, 5-year survival rate = 40%) and without (n = 47, 5-year survival rate = 16.9%) the K-*ras* gene mutation did not statistically differ ($P = 0.62$), nor did survival rates between groups with and without either a *p53* gene mutation or an LOH (n = 17, 5-year survival rate = 0%; n = 15, 5-year survival rate = 40.1%, respectively) ($P = 0.86$). In addition, survival rates between patients with (n = 10, 5-year survival rate = 24%) and without (n = 16, 5-year survival rate = 14.2%) an LOH at the *APC* gene locus did not statistically differ ($P = 0.29$).

DISCUSSION

To our knowledge, *APC* gene abnormalities have not been evaluated in EHBD cancer. In the present study, LOH at the *APC* gene locus was identified in 38.5% of EHBD cancers. However, mutations were undetectable in these same cancers. These results indicate that the prevalence of *APC* gene mutations and LOH in EHBD

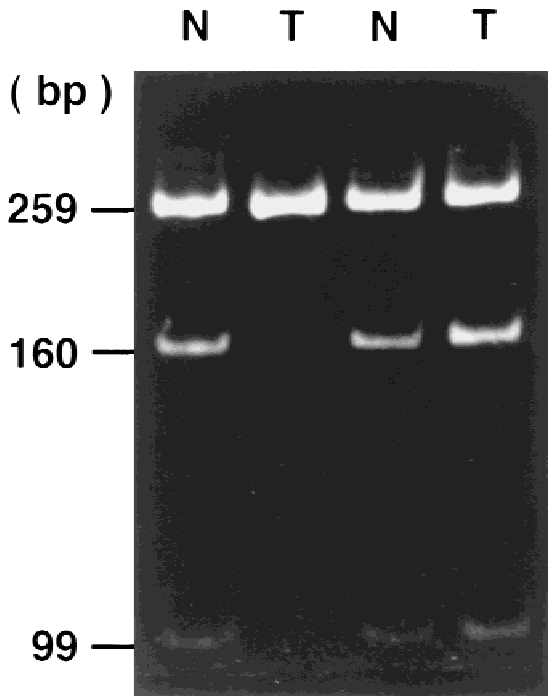

Fig. 3. Polymerase chain reaction restriction fragment length polymorphism analysis of the *p53* gene locus. A 259 bp "uncut" band is an allele lacking the AccII restriction enzyme site in exon 4. Two "cut" bands (160 and 99 bp) represent the allele containing the enzyme site. A sample containing normal DNA migrating as 3 bands is informative. The left normal–tumor pair shows loss of heterozygosity, whereas the right pair retains heterozygosity. N, normal DNA; T, tumor DNA.

cancers differs. There are 2 explanations for this. Firstly, a further unknown tumor-suppressor gene that is proximal to the *APC* gene locus is the true target of allelic loss in EHBD cancer. The interferon regulatory factor 1 (*IRF-1*) gene or other tumor-suppressor genes near *APC* may be the true target of the frequent allelic loss of chromosome 5q in esophageal carcinomas [12,13], gastric cancer [13,14], and non-small-cell lung cancer [15]. Therefore, a deletion at the *APC* gene locus may simply be the result of large deletions on 5q and the *APC* gene may not be important to carcinogenesis of EHBD cancers. Further study is required to identify the major target of the frequent 5q loss identified in EHBD cancers. Secondly, *APC* gene mutations in EHBD cancer might occur on the outside of the mutation cluster region, where somatic mutations are frequently detected in sporadic colorectal tumors [16]. These mutations have been identified in a few patients with familial adenomatous polyposis [17]. We believe that the first explanation is more likely than the second. However, we have not examined the entire coding region of the *APC* gene; therefore, the second explanation cannot be excluded.

The reported overexpression rates of p53 protein in EHBD cancers range between 38% and 66% [18–21]. We immunohistochemically detected p53 protein overexpression in 44.4% of EHBD cancers, and this finding did not correlate with prognosis [22]. In the present study, we detected *p53* gene mutations in 32.7% of EHBD cancers. According to Yoshida et al. [23], the mutational rate of the *p53* gene is very low (1/9, 11.1%) compared with the results of other immunohistochemical studies as well as ours. In the present study, most of the *p53* gene alterations were missense mutations, as demonstrated in other cancers [24], and a characteristic mutation spectrum of the *p53* gene was not found in EHBD cancer. LOH at the *p53* gene locus occurs in 15.6% of EHBD cancers. No other data are available for the LOH at the *p53* gene locus in EHBD cancer. The frequency of p53 allelic loss was lower than the mutational rate. The frequency of LOH may be underestimated when normal stromal and inflammatory cells heavily contaminate specimens. However, we confirmed the cellularity of the cancer cells in tumor specimens before DNA extraction. The frequency of LOH at the *APC* locus was 38.5% in the same DNA samples. The quality of the tumor samples was suitable for LOH analysis. The mechanism of *p53* gene inactivation supposedly consists of the dominant negative effect [25] and alteration of both alleles (2-hit theory) [26]. The present study revealed mutations in all (in codons 147, 175, 229, 242, and 265) of the 5 samples with LOH (100%). Twelve (in codons 157, 158, 212, 239, 248, 265, and 273) of 27 samples without allelic loss (44.4%) carried a mutation in *p53*. Milner and Medcalf [27] demonstrated the ability of particular p53 mutated alleles (in codons 151, 247, and 273) to drive wild-type protein into the mutant conformation during translation. The mutant protein exerted a dominant negative effect in the presence of wild-type p53 but did not gain function in its absence [25]. In the missense mutated samples without LOH, some of these mutant p53 proteins acted in a dominant negative fashion.

The present study showed that the frequency of K-*ras* gene mutations was 9.6% in EHBD cancers. The reported rates of K-*ras* mutations in EHBD cancer range from 8.3% to 100% [28–35]. These variations are probably caused by the methodology applied to PCR analysis. Two-step PCR [30] has proven to be the most sensitive means of detecting K-*ras* gene mutations. However, whether extremely minor tumor populations identified by 2-step PCR in clinically proven cancers that have acquired various genetic alterations is of clinical significance remains questionable.

The present study identified K-*ras*, *p53*, and *APC* gene alterations in early and advanced cancers. Thus, these gene aberrations occur relatively early in the progression of EHBD cancer. Matsubara et al. [36] have detected K-*ras* gene mutations not only in cancerous epithelium but also in hyperplastic, metaplastic, and inflammatory epithelium of the bile duct among patients with pancreatobiliary maljunction. Genetic studies of precancerous

TABLE III. Relationship between Clinicopathological Factors and Aberrations of K-ras, p53, and APC Genes

	Mutations			LOH	
	K-ras	p53	APC	p53	APC
pT					
Tis and T1	37.5% (3/8)[a]	50% (4/8)	0% (0/8)	28.6% (2/7)[c]	66.7% (4/6)
T2 and T3	4.5% (2/44)[b]	29.5% (13/44)	0% (0/44)	12% (3/25)	30% (6/20)
Lymph node metastasis					
Positive	5.9% (1/17)	35.3% (6/17)	0% (0.17)	18.2% (2/11)	33.3% (3/9)
Negative	11.4% (4/35)	31.4% (11/35)	0% (0/35)	14.3% (3/21)	41.2% (7/17)

[a]positive-total samples, [b]$p = 0.02$, [c]positive/informative samples.

lesions require understanding of the pathogenesis of EHBD cancer.

Malats et al. [32] reported that K-*ras* mutations constitute a prognostic indicator among patients with cancer of the extrahepatic bile system. However, Ohashi et al. [33] reported no demonstrable correlation between K-*ras* mutation and prognosis in patients with EHBD cancer. K-*ras, p53,* and *APC* gene abnormalities in the present study did not correlate with malignant phenotypes (pT, pN, and prognosis) associated with EHBD cancer. More patients should be evaluated to determine the usefulness of these genes as prognostic markers in EHBD cancer.

In conclusion, no mutations in the mutation cluster region of the *APC* gene were found, although APC allelic losses were found in 38.5% of EHBD cancers. Our results suggest that an unknown suppressor gene on 5q, other than the *APC* gene, may be responsible for EHBD cancer.

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