

K-*ras* Mutations in Gallbladder Carcinomas in Patients with Anomalous Arrangement of the Pancreaticobiliary Duct

Takao ITOI^{1,2}, Hidenobu WATANABE¹, Tatsuya YOSHIKAWA³, Yoichi AJIOKA¹, Ken NISHIKURA¹ and Toshihiko SAITO²

¹First Department of Pathology, Niigata University School of Medicine, Niigata, ²Fourth Department of Internal Medicine, Tokyo Medical College, Tokyo, ³Department of Gastroenterology Surgery, Tokyo Women's Medical College, Tokyo, Japan

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Summary. Mutation of the K-*ras* codon 12 was examined in 17 gallbladder carcinomas and 14 nonneoplastic gallbladder mucosa in patients with anomalous arrangement of the pancreaticobiliary duct (APBD), using nested polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. In addition, we investigated the relationship between the K-*ras* mutation and cellular proliferation using Ki-67 (MIB1) monoclonal antibody. Mutation of K-*ras* codon 12 was found in 7 of 17 (41.2%) gallbladder carcinomas but not in nonneoplastic gallbladder mucosa. There was neither a correlation between the presence of the K-*ras* mutation and the size or depth of invasion of the carcinoma, nor any statistically significant difference in cellular proliferation of the same grade of cytologic atypia between tumors with and without K-*ras* mutation (Ki-67 labeling index, high grade; $35.2 \pm 13.3\%$ and $31.6 \pm 13.9\%$, low grade; $14.2 \pm 4.9\%$ and $21.2 \pm 12.8\%$, respectively). This indicates that mutation of K-*ras* codon 12 may be involved in the development of gallbladder carcinomas preferentially in patients with APBD, but not in the progression of the Ki-67 labeling index, tumor size, or tumor invasion.

Key words—K-*ras*, cell-proliferative activity, APBD.

INTRODUCTION

Anomalous arrangements of the pancreaticobiliary ducts (APBD) result in a condition where the common bile duct and main pancreatic duct join upstream to the Oddi's sphincter, especially involving non-dilatation of the bile duct, and are frequently

associated with gallbladder carcinomas.¹⁻³⁾ The results of recent clinicopathologic and experimental studies on patients with APBD have suggested that, based on the reflux and stasis of pancreatic juice in the gallbladder, activating pancreatic enzymes, induces diffuse mucosal hyperplasia with or without associated acute inflammation. This hyperplasia may be related to the pathogenesis of well-differentiated carcinomas of the gallbladder.^{3,4)} These studies also suggest that there may be a different genetic pathway for the development of gallbladder carcinomas in patients with APBD and those without APBD.

Mutation of the K-*ras* gene, which encodes a highly conserved protein known as P21 and is thought to control some mechanisms of cell growth and differentiation,^{5,6)} has been found in a wide variety of tumors.⁷⁻¹⁵⁾ More than 80% of pancreatic carcinomas^{7,10-12)} have an activating K-*ras* point mutation in codon 12. Moreover, K-*ras* mutation was found in 62.5% (10 of 16) metaplastic pancreatic epithelia.¹⁶⁾ The incidence of the K-*ras* mutation in gallbladder carcinomas of patients without APBD is 0-55%.⁷⁻⁹⁾ Hence, there may be a high incidence of K-*ras* gene mutation in gallbladder carcinomas of patients with APBD than without APBD. However, to our knowledge, there are no reports in English addressing this possibility.

In this study, we examined the K-*ras* gene mutation in codon 12 in gallbladder carcinomas and corresponding nonneoplastic epithelia of the gallbladders of patients with APBD. Furthermore, we investigated cellular proliferation via the Ki-67 labeling index (LI) in order to determine the relationship between the K-*ras* mutation and cellular proliferation.

Correspondence: Takao Itoi, M.D., First Department of Pathology, Niigata University School of Medicine, Asahimachi-dori 1, Niigata 951, Japan.

MATERIALS AND METHODS

Seventeen primary gallbladder carcinomas associated with APBD diagnosed by endoscopic retrograde cholangiopancreatography and/or endoscopic ultrasonography, all being surgically resected between 1982 and 1994, were obtained from the archives of the First Department of Pathology, Niigata University and the Department of Gastroenterology, Tokyo Women's Medical College. These carcinomas consisted of 9 intramucosal and 8 invasive lesions. Histologic diagnosis was based on examination of hematoxylin-eosin (H & E) stained sections and Ki-67 and p53 immunostained sections, the last of which is a useful marker for cancer diagnosis.¹⁷⁾ Tumor size and depth of invasion was also diagnosed by H & E sections from 4 mm wide consecutive serial paraffin-embedded blocks of the entire resected specimens, with mapping of histopathologic findings on color prints. Histologic classification was based on the guidelines of the Japanese Society of Biliary Surgery.¹⁸⁾

As in the pancreas¹⁶⁾, different *K-ras* mutations were investigated in the nonneoplastic and metaplastic gallbladder epithelia surrounding the tumors. The nonneoplastic and metaplastic epithelia were categorized according to the World Health Organization¹⁹⁾ and Watanabe et al.²⁰⁾ as follows: 1) the proper epithelium is nonmetaplastic gallbladder epithelium; 2) pseudopyloric gland metaplasia of the epithelium contains glands that (a) form small lobules which are usually confined to the lamina propria, (b) are lined by columnar cells with basal nuclei, and (c) have a vacuolated cytoplasm and are similar in appearance to pyloric glands of the stomach; 3) intestinal metaplasia of the epithelium, when fully developed, consists of goblet cells, columnar cells with brush borders, Paneth cells, and endocrine cells, although usually only goblet cells are seen; and 4) surface mucous cell metaplasia of the epithelium contains cells that have vesicular, clear, supranuclear cytoplasm which are filled with sialomucin.²⁰⁾ Hyperplasia of proper and metaplastic epithelia was found in 62.5% (5/8) and 0% (0/6), respectively, of gallbladders in this study. The carcinomas were divided into low and high grades based on cytologic atypia according to our previous reports.^{17,20)}

DNA preparation

DNA extraction from paraffin sections was performed according to the following protocol. Serial sections 3–4 μm and 10 μm thick were prepared. The 3–4 μm -thick sections were stained with H & E or im-

munostained with Ki-67 or p53. Three 10- μm thick sections were deparaffinized with xylene and cleared with ethanol. The tumors and the normal mucosa components were cut from the sections by a surgical knife under a microscope and placed in separate sterile Eppendorf tubes with 300 μl HMW buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA). The tubes then were placed in a heat block (Wakamori Co., Ltd, MH-36, Tokyo, Japan) at 95°C for 15 min. Proteinase K (20 mg/ml) and 10% SDS were subsequently added and the samples were allowed to incubate at 48°C for 24 h. The nucleic acid was purified by organic extraction with phenol/chloroform and then chloroform. It was then precipitated with ethanol, washed, dried, and resuspended in 50 μl of Tris-EDTA. Two microliters of each sample of DNA were used for polymerase chain reaction (PCR) amplification.

Detection of *K-ras* codon 12 mutations

Mutation at codon 12 of *K-ras* was detected by nested PCR-restriction fragment length polymorphism (nested PCR-RFLP) analysis according to the method by Ooshima et al.²¹⁾ One microliter of the DNA solution was used as a template. PCR amplification was performed with the same primers and under the same conditions as previously described²¹⁾ except that the annealing was carried out for 1.5 min. Each amplification included a normal control (normal human placenta DNA), a negative control (no template DNA), and a positive control (AsPcl pancreas cancer cell line DNA that contains a homozygously mutant *K-ras* codon 12; GTT to GAT). A second reaction mixture (10 μl) was incubated overnight with 1 μl of *Mva* I (Takara Co., Ltd. Kyoto, Japan) at 37°C and then electrophoresed in a 4.0% Nu Sieve agarose gel (FMC Bioproducts, Rockland, ME, USA). It was stained with ethidium bromide to confirm the success of the amplification. Any PCR amplification products containing mutation of codon 12 (106-base pair fragment) alone were considered mutations.

Immunostaining

Immunohistochemical staining was performed by a streptavidin-biotin immunoperoxidase method. Four micrometer-thick paraffinized tissue sections were placed onto poly L-lysine-coated glass slides and air dried at room temperature. The sections were deparaffinized and rehydrated, then heated in a microwave oven (Hitachi, MR-M220, Tokyo, Japan) at 500 W (3 min. \times seven) in a citrate-buffer to retrieve antigenic activity. The sections then were allowed to

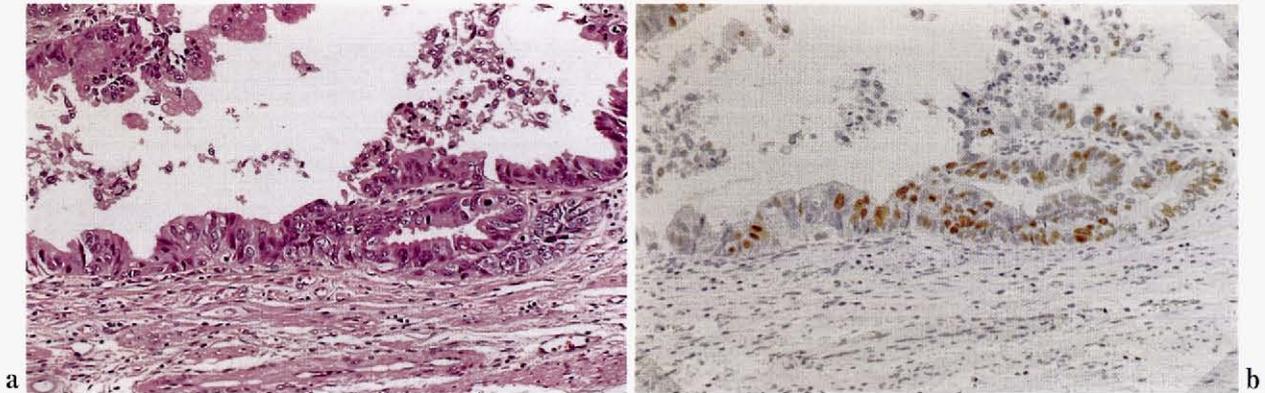


Fig. 1. a. An intramucosal gallbladder carcinoma with high grade cytologic atypia and without a *K-ras* mutation demonstrating swollen, round to oval nuclei with speckled chromatin and distinct nucleoli. (H & E, Case no. 1, $\times 48$) **b.** Ki-67 immunoreactive cells are diffusely distributed in the area of the carcinoma corresponding to that of Fig. 1a. (Ki-67 immunostain, $\times 48$)

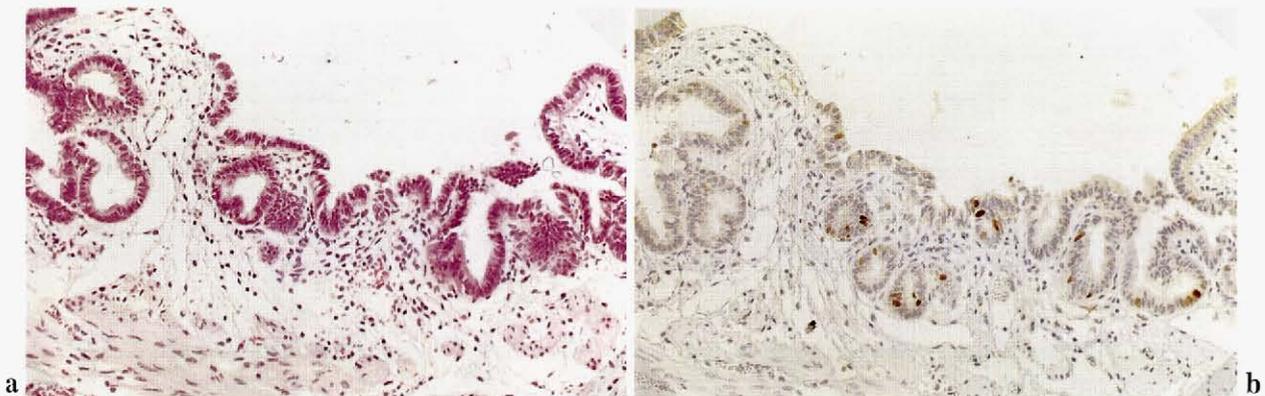


Fig. 2. a. An intramucosal gallbladder carcinoma with low grade cytologic atypia and with a *K-ras* mutation. (H & E, Case no. 11, $\times 24$) **b.** Ki-67 immunoreactive cells are sporadically distributed in the carcinoma in this area, corresponding to that of Fig. 2a. (Ki-67 immunostain, $\times 24$)

cool for 60 min at room temperature.²²⁾ Endogenous peroxidase activity was inhibited by incubation of the slides with 0.3% hydrogen peroxidase in methanol for 20 min at room temperature. After blocking non-specific binding with 10% normal rabbit serum, the sections were incubated with a 1 : 50 dilution of Ki-67 antibody (MIB1; Immunotech, Marseille, France) for 2 h at room temperature with a 1 : 200 dilution of p53 antibody (Ab-2; Oncogene Science Inc. Manhasset, NY, USA) for 1 h at room temperature. The sections then were incubated with biotinylated rabbit anti-mouse immunoglobulin for 20 min and finally with the streptavidin-peroxidase complex (Histofine SAB-PO Kit, Biogenex Laboratories, Dublin, CA, USA) for 30 min. Careful rinses were performed with several changes of phosphate buffered saline between each stage of the procedure. Color reaction products were

developed with diaminobenzidine (reaction time 5–6 min). The sections were lightly counterstained with hematoxylin and then coverslipped.

Immunohistochemical analysis

Ki-67-immunoreactive cells were defined as cells containing a brown reaction product in their nucleus, regardless of the staining intensity. The Ki-67LI was determined by counting 500–643 nuclei in an area of the section from where DNA was extracted and immunoreactivity was most homogeneous. The presence of acute inflammation in the carcinoma also was examined because cellular proliferation may be increased in such regions.

Table 1. K-*ras* codon 12 mutation in gallbladder carcinomas of patients with APBD

No. (NG. No)	Age/Sex (yr)	Size of tumor (mm)	Depth of invasion¶	Histologic type§	Ki-67LI†	Grade of cytologic atypia	Acute inflammation#	K- <i>ras</i> codon 12 mutation#	
								*Tumor (phenotype)	*Non neoplasia (phenotype)
1(92-360E2)	54/F	19	m	pap	13.7%(72/524)	Low	–	– (P)	– (P, MH)
2(6038)	60/F	25	m	tub 1	20.4%(113/553)	High	–	– (SMM)	– (P, MH)
3(8723)	57/M	7	m	tub 1	18.3%(94/514)	Low	–	– (SMM)	– (P)
4(8861)	57/M	20	m	tub 1	52.8%(329/623)	High	–	– (SMM)	– (SMM)
5(9671-16)	67/F	11	m	pap	33.2%(211/635)	High	–	– (PMM)	– (PMM)
6(2218)	62/F	30	ss	pap	46.4%(268/578)	Low	+	– (P)	– (P, MH)
7(6995)	57/F	22	ss	tub 1	13.5%(79/584)	Low	–	– (P)	ND**
8(7127)	74/F	15	ss	tub 1	13.2%(66/500)	Low	–	– (PPM)	– (PPM)
9(7932)	73/F	20	si	tub 1	13.1%(77/589)	High	–	– (P)	ND
10(9671-8)	67/F	8	ss	pap	38.3%(202/528)	High	+	– (SMM)	– (P)
11(94-925F2)	64/F	20	m	tub 1	13.8%(79/572)	Low	–	+	– (P, MH)
12(3673)	60/F	35	m	pap	6.4%(32/500)	Low	–	+	– (P, MH)
13(5641-5)	38/F	8	m	pap	18.8%(107/568)	Low	–	+	– (PPM)
14(5641-16)	38/F	10	m	pap	17.7%(109/615)	Low	+	+	– (PPM)
15(2524-28)	62/F	10	ss	por 2	52.1%(335/643)	High	–	+	– (PPM)
16(7076)	63/M	18	ss	pap	34.0%(192/564)	High	–	+	– (SMM)
17(7255-6)	49/F	42	ss	pap	19.5%(102/524)	High	+	+	ND

¶m; mucosa, ss; subserosa, si; infiltrating to adjacent tissue.

§pap; papillary adenocarcinoma, tub 1; well-differentiated adenocarcinoma, por 1; poorly-differentiated adenocarcinoma with medullary growth, por 2; poorly-differentiated adenocarcinoma with scirrhous growth.

†LI; Ki-67 immunoreactive cells/total number of cells counted.

*Normal; nonneoplastic mucosa around carcinoma, P; proper epithelium, PPM; pseudopyloric gland metaplasia, SMM; surface mucous cell metaplasia, MH; mucosal hyperplasia.

**ND; not done.

#+; present, –; absent.

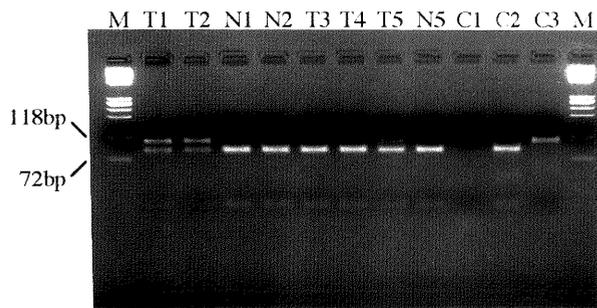


Fig. 3. Nested polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of *K-ras* codon 12 mutations (M: ϕ X174/HaeIII DNA size control). Mva I digests of DNA from 3 carcinomas (T1, T2 and T5; Case no. 11, 13 and 16, respectively) have both normal and mutated DNA (106 bp and 86 bp), while digests of DNA from 2 carcinomas (T3 and T4; Case no. 1 and 5) and nonneoplastic gallbladder mucosa (N1, N2 and N5; Case no. 11, 13 and 16, respectively) demonstrate normal DNA only. The negative control (C1) has no band, while the normal control (C2) has normal DNA only, and the positive control (C3) has mutated DNA only.

Table 2. Correlation of *K-ras* mutation, Ki-67 LI, and grade of cytologic atypia

<i>K-ras</i> mutation	Grade of cytologic atypia (No. of case)	Ki-67 LI (%)	
Present	High (3)	35.2 ± 13.3	NS
	Low (4)	14.2 ± 4.9	
Absent	High (5)	31.6 ± 13.9	NS
	Low (5)	21.2 ± 12.8	

Statistical analysis

Differences in the incidence of *K-ras* gene mutations were evaluated by the X^2 test or Fisher's exact test (two-tailed). Differences in the Ki-67 LI and in tumor size were evaluated by the Mann-Whitney U-test. Probability values of less than 0.05 were considered statistically significant.

RESULTS

K-ras codon 12 mutations were identified in 7 of 17 (41.2%) gallbladder carcinomas (Table 1), but in none of the proper or metaplastic mucosae (Table 1 and

Fig. 3). The incidence of *K-ras* mutation did not differ significantly between tumors with low or high grades of cytologic atypia (44.4% (4 of 9) low grade, 37.5% (3 of 8) high grade). There were also no significant differences when tumor size (*K-ras* mutation (+); 20.4 ± 13.3 mm, (-); 17.7 ± 7.4 mm) or depth of invasion (4 of 9 mucosal carcinomas, 3 of 8 invasive carcinomas) were considered.

There also was no statistically significant difference in cellular proliferation of the same grade of cytologic atypia between tumors with and without *K-ras* mutation (Ki-67 labeling index, high grade; $35.2 \pm 13.3\%$ and $31.6 \pm 13.9\%$, low grade; $14.2 \pm 4.9\%$ and $21.2 \pm 12.8\%$, respectively). (Table 2 and Figs. 1 and 2)

DISCUSSION

Our previous study on *K-ras* mutations in gallbladder carcinomas contained 7 patients with APBD and 33 without APBD, and 14% (1 of 7) of the patients with APBD and 9% (3 of 33) of those without APBD had a *K-ras* mutation.²³⁾ There was no significant difference between these data. In the present study, however, the frequency of *K-ras* mutation in the gallbladder carcinomas of patients with APBD was significantly higher than in those without APBD (7/17, 41.2% vs. 3/33, 9%, $p < 0.05$). Although the reason for the difference between the previous and present data is not clear, it may be explained by the fact that cases with APBD increased in the present study. The high frequency of *K-ras* mutation of gallbladder carcinoma with APBD suggests that activated pancreatic enzymes in the pancreatic juice refluxing into the gallbladder and bile acid alteration due to its reflux may induce *K-ras* mutation.

K-ras mutation was not detected in the non-neoplastic proper or metaplastic mucosae regardless of the presence or absence of mucosal hyperplasia. The nonneoplastic gallbladder mucosa, ie., proper epithelium or mucous metaplastic epithelium, is somewhat similar histologically and immunohistochemically to that of the pancreas. However, recent articles have documented that *K-ras* mutations were identified in 10 of 16 (62.5%) pancreatic metaplastic epithelia¹⁶⁾ and were more frequent in pancreatic carcinomas (greater than 80%).¹⁰⁻¹²⁾ It is not clear why the frequencies of *K-ras* mutations are different between pancreatic ductal epithelium and gallbladder mucosa. However, our results suggest that *K-ras* mutation may be associated more closely with the development of carcinomas in the gallbladders of patients with APBD than in patients without APBD.

Since one function of the *K-ras* may be the control of cellular growth,⁶⁾ activating the *K-ras* gene may be associated with cellular proliferation. In colorectal tumors, the *K-ras* mutation correlated with the elongation of the proliferative zone and a higher Ki-67 LI.²⁴⁾ In the gallbladder carcinomas of patients with APBD, however, we could not detect any association between the *K-ras* codon 12 mutation and increased Ki-67 LI. Since other hot spots for *K-ras* mutations, generally point mutations, are known in codon 13 and 61, it may be possible for mutations to occur at these codons. However, in a previous report on gallbladder carcinoma,⁷⁾ *K-ras* mutation was detected only at codon 12.

In conclusion, the present data suggest that the *K-ras* codon 12 mutation may be more closely associated with the development of gallbladder carcinomas in patients with APBD than in patients without APBD, and is not associated with increased cell proliferation, tumor growth, or tumor invasion.

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