

Analyses of *p53* Overexpression, Aberrant β -Catenin Expression, Microsatellite Instability, and *K-ras* Mutation in Intrahepatic Cholangiocarcinoma with and without Hepatolithiasis

Hiroki SHIMIZU¹, Yoichi AJIOKA², Takashi AKAGAWA³, Koji ODA³, Yuji NIMURA³, Miin-Fu CHEN⁴, Yi-Yin JAN⁴, Shiu-Feng HUANG⁵, Gen WATANABE², Ken NISHIKURA² and Katsuyoshi HATAKEYAMA¹

¹Division of Digestive and General Surgery, Department of Regenerative and Transplant Medicine, ²Division of Molecular and Diagnostic Pathology, Department of Molecular Genetics, Graduate School of Medical and Dental Sciences, Niigata University, Niigata; ³Division of Surgical Oncology, Department of Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan; ⁴Department of Surgery and ⁵Department of Pathology, Chang Gung Memorial Hospital, Lin Kou, Taiwan

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Summary. To determine whether genetic alterations in intrahepatic cholangiocarcinoma (ICC) differ by the association of chronic inflammation induced by hepatolithiasis, we analyzed *p53* protein overexpression, aberrant β -catenin nuclear expression, microsatellite instability (MSI) status, and *K-ras* codon 12 mutation in 20 cases with ICC (13 with hepatolithiasis and seven without hepatolithiasis). Positive frequencies of each variable for ICC with and without hepatolithiasis were: *p53* overexpression, 38% vs 14%; aberrant β -catenin nuclear expression, 23% vs 0%; MSI-high, 0% vs 0%; MSI-low, 13% vs 17%; and *K-ras* mutation, 31% vs 14%. Aberrant β -catenin nuclear expression showed a sporadic pattern in all cases. There were no significant differences in any of the frequencies between ICCs with and without hepatolithiasis. These results suggest that genetic alterations in ICC do not differ according to the presence of chronic inflammation by hepatolithiasis. However, these results may be partly attributable to the small sample number of ICC cases with hepatolithiasis in the present study. Additional studies of ICC with hepatolithiasis and further investigations are required to obtain definitive conclusions. With regard to ICC without hepatolithiasis, the present results imply that alterations of *p53* and *K-ras* may play crucial roles in the carcinogenesis of a subset (30 to 40%) of ICC,

and adenomatous polyposis coli (APC) alterations represented by aberrant β -catenin nuclear expression and defects in the DNA mismatch repair system represented by MSI-high may not contribute to ICC carcinogenesis.

Key words— intrahepatic cholangiocarcinoma, microsatellite instability, *K-ras*, β -catenin, *p53*, hepatolithiasis, carcinogenesis.

INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC), a type of adenocarcinoma arising from the intrahepatic bile ducts, comprises the second most common primary liver cancer after hepatocellular carcinoma in Japan.¹⁾ Most cases of ICC are of unknown etiology and develop in the noncirrhotic liver. However, a number of risk factors have been established, such as fibrocystic diseases,²⁾ extrahepatic biliary atresia,³⁾ inflammatory bowel disease,⁴⁾ gallstones,⁵⁾ parasitic diseases,⁶⁾ thorotrast exposure,⁷⁾ and recurrent pyogenic cholangitis caused by hepatolithiasis.^{8,9,10,11)}

It has recently been shown that the neoplastic transformation of biliary epithelial cells and a malignant progression of ICC are accompanied by a number of genetic and epigenetic alterations of oncogenes and

Correspondence: Yoichi Ajioka M.D., Division of Molecular and Diagnostic Pathology, Department of Molecular Genetics, Graduate School of Medical and Dental Sciences, Niigata University, Asahimachi-dori 1-757, Chuo-ku, Niigata 951-8510, Japan.

Abbreviations – APC, adenomatous polyposis coli; ICC, intrahepatic cholangiocarcinoma; LOH, loss of heterozygosity; MSI, microsatellite instability.

tumor suppressor genes. Genetic alterations, such as the point mutation of *K-ras*¹²⁻²⁰ and *p53*,^{14,16,17,19,21} loss of heterozygosity (LOH)^{17,22,23} and promoter methylation of adenomatous polyposis coli (*APC*),^{24,25} *p53* protein overexpression,^{15,17,18,20,26,27} and nuclear expression of β -catenin,^{28,29,30} have been found in subsets of ICC. The overexpression of *p53* protein is regarded as a result of *p53* gene mutation,³¹ and nuclear β -catenin expression represents an aberrant nuclear accumulation of β -catenin protein, which is allowed by *APC* suppressor gene mutations and may accelerate the Wnt signaling pathway leading to tumorigenesis.³² Furthermore, some ICCs show defects in the DNA mismatch repair (MMR) system occasionally demonstrated as a microsatellite instability high phenotype.^{19,20} It is of interest whether these genetic and/or epigenetic alterations have any relation to differences in the associated risk factors of ICC. To date, however, there have been no studies from this view point with the exception of those by Tsuda et al.¹² and Kiba et al.¹⁴ In these reports, it is demonstrated a marked contrast in the incidence of *K-ras* point mutation between ICC in Japanese patients and those in northeastern Thailand where the liver fluke *Opisthorchis viverrini* is endemic.

Among the various risk factors for ICC carcinogenesis, we focused on hepatolithiasis and investigated whether the association of this risk factor makes any difference in *p53* overexpression, aberrant β -catenin nuclear expression, microsatellite instability (MSI) status, or *K-ras* codon 12 point mutation in ICC.

MATERIALS AND METHODS

Specimens

Formalin-fixed, paraffin-embedded tissues of 20 ICCs were examined. Thirteen out of 20 patients were not associated with hepatolithiasis and underwent surgical operation at Niigata University Medical and Dental Hospital (Niigata), while the remaining seven were associated with hepatolithiasis (Fig. 1) and were obtained from Chang Gung Memorial Hospital, Taiwan. Serial sections, three 4 μ m-thick and five 10 μ m-thick, were prepared from the representative specimen. The 4- μ m sections were used for hematoxylin and eosin staining and immunohistochemistry, and the 10- μ m sections were used for DNA extraction. Histological examination was performed according to the proposals of the Liver Cancer Study Group of Japan.³³ All cases had invasive carcinomas associated with and without non-invasive intraductal components. Histological typing, immunohistochemical evaluation, and DNA extraction were performed at the evidently invasive part of tumor.

Immunohistochemistry

Immunohistochemistry was performed using the streptavidin-biotin immunoperoxidase method (Histofine SAB-PO kit; Nichirei, Tokyo). Monoclonal antibodies used were 17C2 (Novocastra, Newcastle upon Tyne, UK) against β -catenin protein and PAb1801 (Oncogene Science, Manhasset, NY, USA) against *p53* protein. The sections for *p53* immunostaining were counterstained with hematoxylin and those for β -catenin were also counterstained with 2% methyl green solution (Chroma, Kongen, Germany). The staining pattern of *p53* was classified as follows: 1) negative; 2) a few scattered positive cells; 3) a localized aggregation of positive cells, and 4) diffusely positive cells; the latter two patterns were regarded as an overexpression.³⁴ β -catenin normally functions as a cell adhesion molecule, and the basolateral membrane of epithelial cells shows a positive immunostaining for it.³⁵ In each sample, nuclear β -catenin staining was evaluated as an aberrant expression. Nuclear immunostaining patterns were classified as follows: 1) negative; 2) sporadic; very few scattered positive cells; 3) clustered, positive cells clustered in focal areas; and 4) diffuse; diffuse distribution of positive cells.³⁶

DNA preparation

Cancer tissue and non-neoplastic bile duct tissue in each sample were dissected under microscopic guidance, and DNA was extracted using a DNA Isolator PS Kit (Wako Pure Chemical Industries, Ltd., Osaka) as described previously.³⁷

Analysis of *K-ras* mutations

K-ras mutations at codon 12 were analyzed by a nested PCR restriction fragment length polymorphism analysis as described by Ooshima et al.³⁸

Analysis of MSI

Fluorescent dye-labeled PCR amplification was performed using the five microsatellite makers (*BAT-25*, *BAT-26*, *D2S123*, *D5S346*, and *D17S250*) recommended by the National Cancer Institute Workshop.^{39,40} Fluorescent dye-labeled and unlabeled primers were obtained from Applied Biosystems Japan Ltd. (Tokyo); the 5' oligonucleotide was end-labeled with 6FAM (*BAT-25*, *D2S123*), VIC (*BAT-26*, *D17S250*), or NED (*D5S346*) fluorescent dyes. PCR amplification was performed using a Temp Control System PC-700 (Astec Co., Ltd., Fukuoka) in reaction volumes of 30 μ l containing 100 ng of DNA, 0.75 U of AmpliTaq Gold (Applied Biosystems Japan Ltd., Tokyo), and 10 pmol

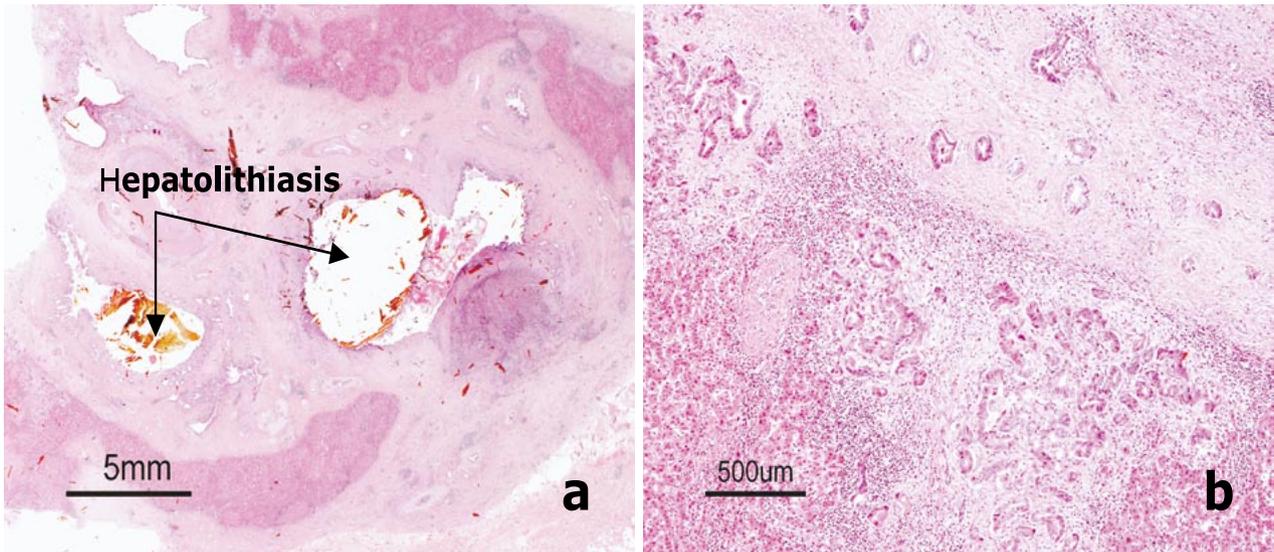


Fig. 1. Intrahepatic cholangiocarcinoma (ICC) with hepatolithiasis. **a.** Arrows indicate hepatolithiasis. HE x 1 **b.** An adenocarcinoma can be seen growing in abundant fibrous stroma. HE x 4 (Original magnification)

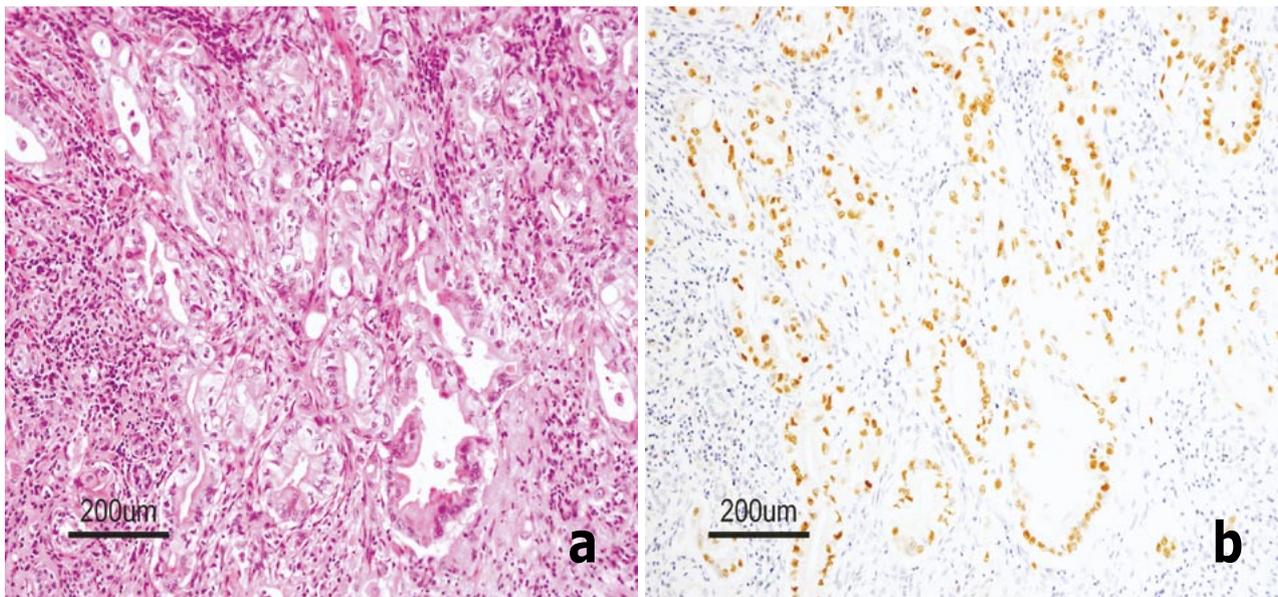


Fig. 2. ICC with overexpression of *p53* protein. **a.** G2 ICC. HE x 10 **b.** Nuclei of the cancer tissue are diffusely positive for *p53* immunostaining. x 10 (Original magnification)

Table 1. Results of immunohistochemistry for *p53* and β -catenin, microsatellite instability, and *k-ras* codon 12 mutation in 20 intrahepatic cholangiocarcinomas

Case no.	Histological grade	<i>p53</i> overexpression	Aberrant β -catenin nuclear expression	Microsatellite instability	<i>K-ras</i> codon 12 mutation
Without hepatolithiasis (n = 13)					
1	G1 > G2	–	–	MSS	–
2	G1	–	–	MSI-L (<i>D2S123</i>)	+
3	G2 > G3	+	Sporadic	ND	–
4	G2 > G3	+	–	ND	–
5	G1 > G2	+	–	MSS	–
6	G1 > G2	–	–	ND	+
7	G2 > G1	–	–	ND	–
8	G1 > G2	–	Sporadic	MSS	+
9	G1 > G3	–	–	MSS	–
10	G3	+	–	MSS	–
11	G3	–	–	ND	–
12	G2 > G3	–	–	MSS	–
13	G2 > G3	+	Sporadic	MSS	+
With hepatolithiasis (n = 7)					
14	G1 - G2	–	–	MSS	–
15	G2 - G3	–	–	MSS	–
16	G1	–	–	ND	–
17	G3	–	–	MSI-L (<i>D2S123</i>)	–
18	G1	–	–	MSS	–
19	G1	–	–	MSS	+
20	G2 - G3	+	–	MSS	–

G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; MSS, microsatellite stable; MSI-L, microsatellite instability-low; ND, not detected.

Table 2. Genetic alterations in 20 intrahepatic cholangiocarcinomas according to the presence or absence of hepatolithiasis

	<i>p53</i> overexpression	Aberrant β -catenin nuclear staining	MSI-H	MSI-L	<i>K-ras</i> codon 12 mutation
Without hepatolithiasis (n = 13)	5	3	0	1	4
With hepatolithiasis (n = 7)	1	0	0	1	1
Total (n = 20)	6	3	0	2	5

MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low.

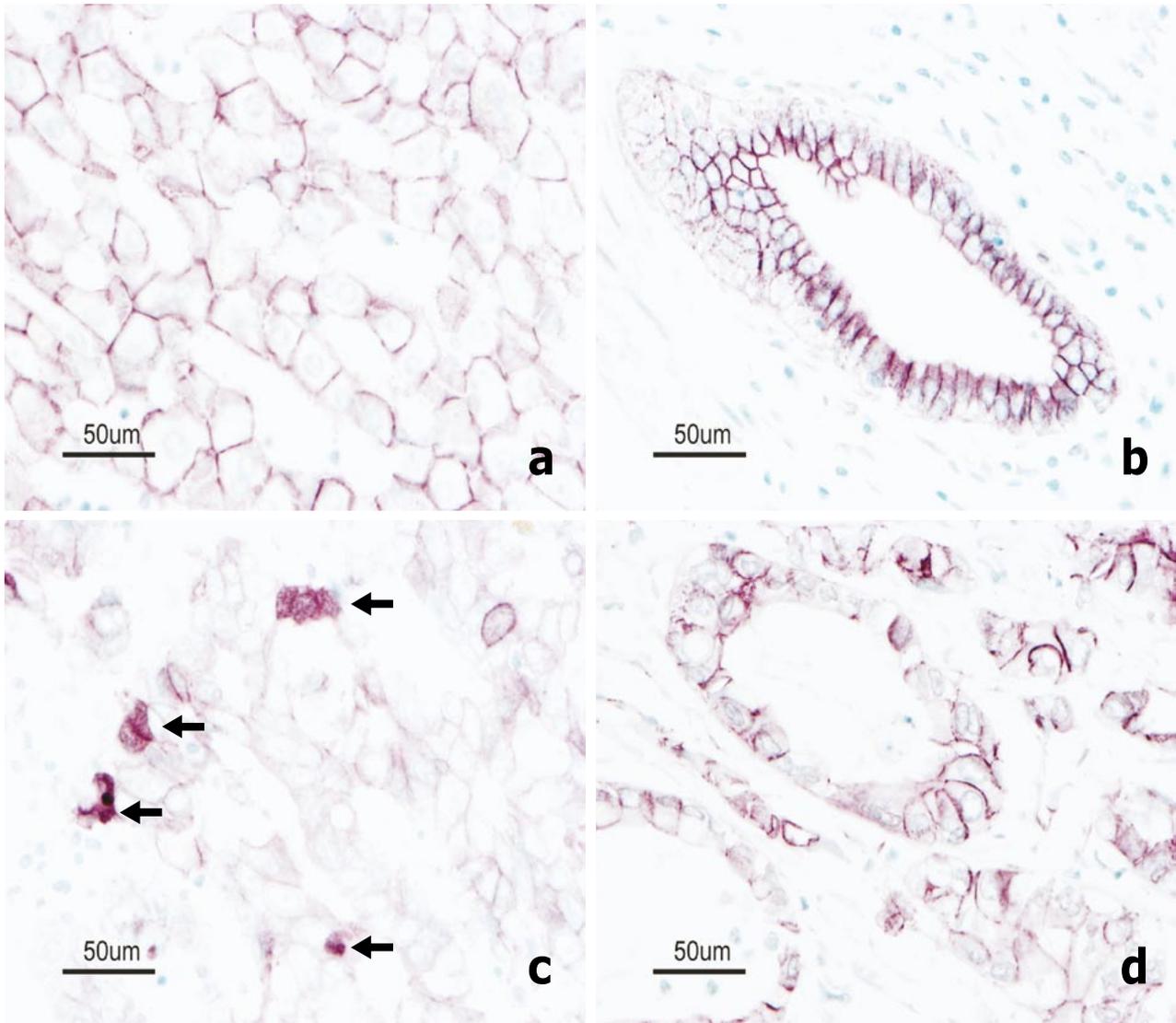


Fig. 3. β -catenin immunostaining. **a.** Basolateral membrane expression within the normal hepatocytes. x 40 **b.** Epithelial cells of the bile duct. x 40, and **c.** ICC x 40 **d.** ICC with aberrant β -catenin nuclear expression. x 20 Arrows indicate a few scattered positive nuclei.(Original magnification)

of each primer. Annealing temperatures were optimized for each marker. Aliquots of 1.0 μ l of each fluorescent dye-labeled PCR product were combined with 12 μ l of formamide and 0.5 μ l of the GENESCAN 350 [ROX] size standard (Applied Biosystems Ltd., Tokyo), and the results were analyzed on an ABI PRISM 310 Genetic Analyzer using GeneScan Analysis Software (Applied Biosystems Ltd., Tokyo).

The presence of MSI was determined by PCR amplification of the five microsatellite markers. MSI was defined as the presence of novel peaks that were not found in non-neoplastic tissue. A tumor was defined as having high microsatellite instability (MSI-high) if more

than one of the five loci analyzed showed unequivocal instabilities; it was defined as having low microsatellite instability (MSI-low) if only one locus showed instability; and it was defined as microsatellite stable if no microsatellite instability was found.³⁹⁾

Statistical analysis

Statistical analyses were performed by Fisher's exact test. All tests were two-sided and a *P* value less than 0.05 was considered to be statistically significant.

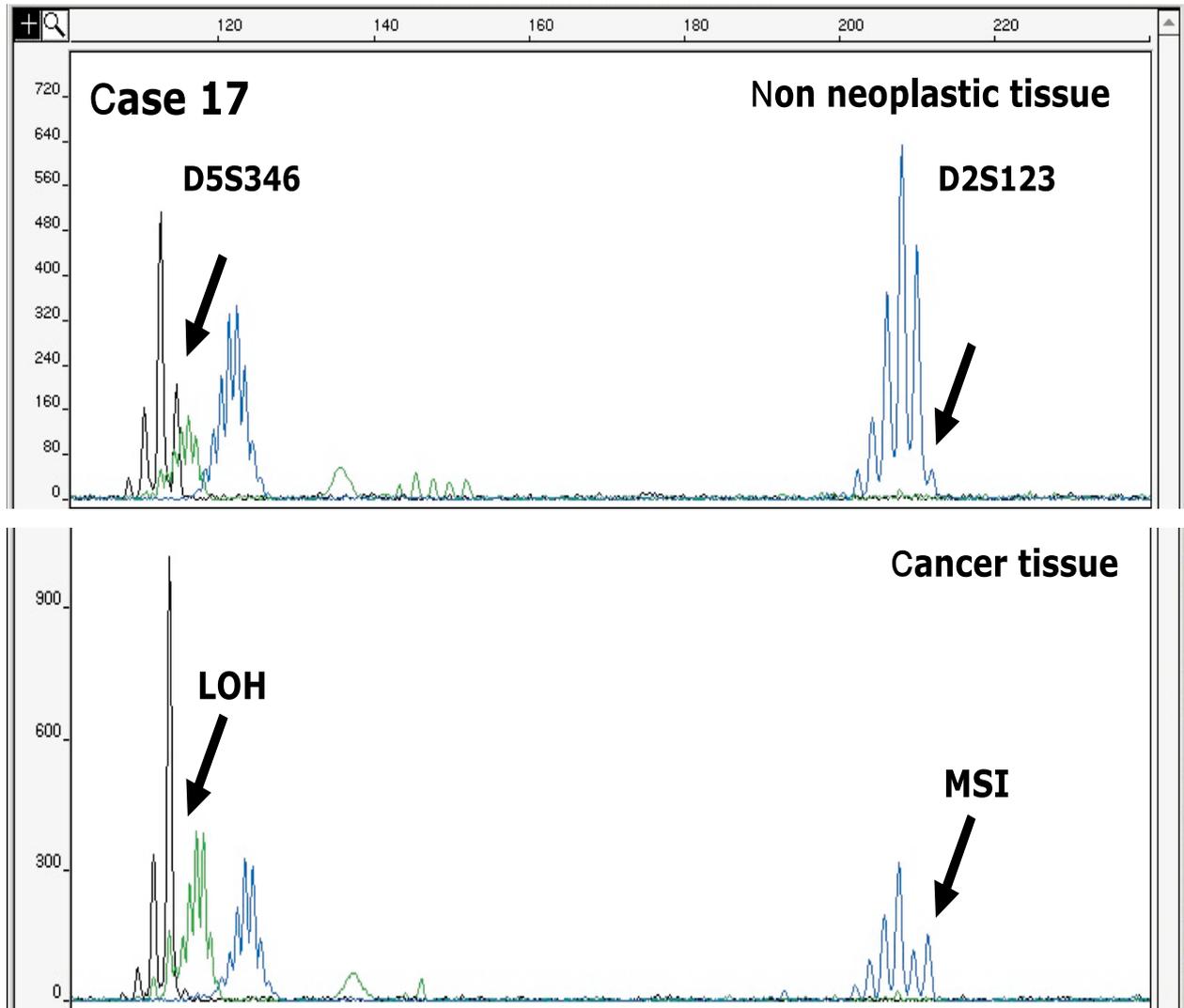


Fig. 4. Fragment pattern of case 17 (**Table 1**) showing microsatellite instability at one locus (*D2S123*) within the cancerous tissue.

RESULTS

Results of immunohistochemistry, MSI, and *K-ras* mutation are shown in Table 1 and summarized in Table 2. There was no significant difference in histological type of ICC between cases with and without hepatolithiasis.

The overexpression of *p53* protein was detected in 6/20 (30%) of ICCs (Fig.2), and the frequency was not significantly different between the ICC with and without hepatolithiasis (5/13, 38% and 1/7, 14%, respectively). In the normal tissue, β -catenin expression was seen along the basolateral membranes of the hepatocytes (Fig.3a) and epithelial cells of the bile ducts (Fig.3b), and no nuclear staining was observed. Most ICCs showed a basolateral membrane expression similar to normal tissue

(Fig.3c), and a aberrant β -catenin nuclear expression was seen only in 3/20 (15%) of ICCs (Fig.3d), all of these were classified as a sporadic pattern. There was no significant difference in the frequency of a aberrant β -catenin nuclear expression between the ICC with and without hepatolithiasis (3/13, 23% and 0%, respectively).

The analysis of MSI was successful in 8/13 and 6/7 ICCs with and without hepatolithiasis, respectively. Template DNA failed to amplify from paraffin sections in five of 13 and one of seven cases in each group. MSI was detected in one case in each group (cases two and 17) (Fig.4), and these were both an MSI-low phenotype. There was no significant difference in the frequency of MSI-low phenotypes between ICCs with and without hepatolithiasis (1/8, 13% and 1/6, 17%, respectively). *K-ras* codon 12 mutation was found in 5/20 (25%) of

ICCs, and the frequency was not significantly different between the ICC with and without hepatolithiasis (4/13, 31% and 1/7, 14%, respectively).

There were no significant associations between histological grade and each variant examined.

DISCUSSION

Although most cases of ICCs are of unknown etiology, several well-known risk factors have been established.²⁻¹¹ However, most studies investigating genetic or epigenetic alterations in ICC have not considered the presence or absence of these background risk factors. The frequencies of alterations in oncogenes and tumor suppressor genes in ICCs reported in earlier studies indicate very wide ranges: 4.6 to 50% for *K-ras* codon 12 point mutation,¹²⁻²⁰ five to 33% for *p53* alterations,^{14,16,17,19, 21} zero to 31% for *p53* protein overexpression,^{15,17,18,20,26,27} and zero to 68.8% for LOH of APC.^{17,22,23} These wide ranges of genetic alterations were detectable in ICCs, may be attributed to differences in etiology or pathogenesis. Tsuda et al.¹² and Kiba et al.¹⁴ investigated the frequency of *K-ras* mutation of ICCs from patients in Japan and in northeastern Thailand. They demonstrated that *K-ras* mutation was markedly infrequent for patients in northeastern Thailand where the liver fluke *Opisthorchis viverrini* is endemic. This result suggests that differences in etiology or background risk factors can be associated with differences in the type of genetic or epigenetic alterations occurring during the carcinogenesis of ICCs.

On the other hand, the association of chronic inflammation with a variety of carcinomas is well recognized: e.g., colorectal cancers with ulcerative colitis,⁴¹ gastric cancer with atrophic gastritis,⁴² gallbladder cancer with chronic cholecystitis and cholelithiasis,⁴³ and Barrett's adenocarcinoma with reflux esophagitis.⁴⁴ The repetitive cycles of inflammation and repair that characterize these inflammatory diseases are thought to generate oxygen molecules, which randomly damage epithelial DNA causing oncogenic mutations and progression to malignancy.^{45,46} The association of hepatolithiasis with ICCs may be along the same carcinogenetic line, namely in that persistent pyogenic cholangitis stems from hepatolithiasis, and that it may induce various oncogenic mutations in bile duct epithelial cells. With regard to the molecular pathways of carcinomas associated with chronic inflammation, it is known that ulcerative colitis-related colorectal carcinoma shows different genetic alterations to sporadic cases: e.g., APC mutation/LOH, *K-ras* mutation, DCC mutation/LOH, and β -catenin dysregulation are more infrequent and the alteration of *p53* is an earlier event in carcinogenesis.⁴⁷ Therefore, we hypothesize that ICCs with and without hepatolithiasis are similar to ulcerative colitis-related colorectal carcinoma, and these can show different types of

genetic alteration.

In contrast to our hypothesis, we found no significant differences in *p53* overexpression, aberrant β -catenin nuclear expression, MSI status, or *K-ras* codon 12 point mutation between ICCs with and without hepatolithiasis. Our results suggest that genetic alterations in ICCs, at least as far as those examined here, would not differ with the presence of chronic persistent inflammation induced by hepatolithiasis. However, our failure to identify such differences may be partly attributable to the small sample size of ICCs with hepatolithiasis ($n = 7$). Additional studies of ICCs with hepatolithiasis are required. Further detailed investigations taking account of the duration and degree of inflammation induced by hepatolithiasis, and a comparison of the genetic alterations of the intraductal components of ICCs — which are regarded as an early stage of carcinogenesis, with and without hepatolithiasis, are needed for any definitive conclusions.

With regard to genetic alterations in ICCs without hepatolithiasis, our results imply that the alteration of *p53* represented by the overexpression of the *p53* protein and *K-ras* mutation may play a crucial role in the carcinogenesis of a subset (30 to 40%) of ICCs. On the other hand, APC alterations and genetic defects in the DNA mismatch repair system may not contribute to ICC carcinogenesis. APC alterations are thought to result in increases in cytoplasmic β -catenin levels and translocation and accumulation in the nuclei.³² In this study, while an aberrant β -catenin nuclear expression was detected in 23% of ICCs without hepatolithiasis, the staining pattern was sporadic, so that it cannot be concluded that the nuclear accumulation of β -catenin played a role in either the initiation or progression of the tumor. MSI-high alone is considered the defect in the DNA mismatch repair system,⁴⁸ and there were no tumors with the MSI-high phenotype among the cases of ICC examined in the present study.

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