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RAPID COMMUNICATION

Genetic changes of *p53*, K-*ras*, and microsatellite instability in gallbladder carcinoma in high-incidence areas of Japan and Hungary

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Abstract

AIM: To disclose geographic differences in genetic changes involved in gallbladder carcinogenesis between two distinct high-incidence areas of Japan and Hungary.

METHODS: We examined 42 cases of gallbladder carcinoma: 22 Japanese and 20 Hungarian cases. *p53* mutations at exons 5 to 8 and K-*ras* mutations at codon 12 were tested by direct sequencing. Microsatellite instability was determined from fluorescent dye-labeled PCR amplifications of five-microsatellite markers (*BAT-25*, *BAT-26*, *D2S123*, *D5S346*, and *D17S250*).

RESULTS: Mutations of *p53* were detected in 11 of 22 Japanese cases and 6 of 18 Hungarian cases (11/22 *vs* 6/18, $P = 0.348$). Transition at CpG sites was found in none of 11 Japanese cases and 2 of 6 Hungarian cases; the difference was marginally significant (0/11 *vs* 2/6, $P = 0.110$). K-*ras* mutations were detected in only one of the Hungarian cases. Eight of 19 (42.1%) Japanese cases were MSI-high (presence of novel peaks in more than one of the five loci analyzed), whereas only 1 of 15 (6.7%) Hungarian cases was MSI-high ($P = 0.047$).

CONCLUSION: It appears that the *p53* mutations and MSI differ in patients with gallbladder carcinoma between two distinct high-incidence areas. Geographic variation might exist in the process of gallbladder carcinogenesis.

Key words: Gallbladder; Gallbladder Neoplasms; K-*ras*; Microsatellite instability; *p53*

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INTRODUCTION

Although considerable progress has been made regarding the molecular pathogenesis of human neoplasms such as colorectal carcinoma[1,2], pancreatic carcinoma[3,4], and breast carcinoma[5,6], there is only limited information about the genetic changes involved in gallbladder carcinogenesis[7,8]. Gallbladder carcinoma shows striking geographic and ethnic variation[9,10]. The high-incidence areas are scattered throughout the world: Latin America, Eastern Europe, northern India, and Japan[9-13]. The highest mortality rate of gallbladder carcinoma in the world, 35 per 100 000 inhabitants, is found in Southern Chile[9]. Japanese standardized mortality rates for gallbladder carcinoma were world's second highest for males and fifth highest for females in 1996 with a steady increase in incidence (up to 5 per 100 000)[9,10,14]. On the other hand,

Zatonski reported that Hungarian mortality rates of this tumor were the highest in Europe (4 per 100 000 males and 7 per 100 000 females)[13]. This marked geographic variation implies that a combination of genetic and environmental etiologic factors affects the process of carcinogenesis of the gallbladder[10]. Previously, we revealed the geographic variation of gallbladder carcinogenesis between Japan and Chile (a representative of Latin American countries), both of which were known as distinct high-incidence countries, in terms of the *p53* mutational spectra[14]. Only a few other investigators also have reported such geographic and ethnic differences in genetic changes of this tumor[15-17]. Thus, there has been a paucity of evidence regarding the geographic diversity of genetic changes involved in gallbladder carcinogenesis.

The aim of this study was to disclose geographic differences in genetic changes involved in gallbladder carcinogenesis by comparing gallbladder carcinomas from two distinct high-incidence areas, Japan and Hungary (a representative country of Eastern Europe), in terms of *p53* mutations, K-ras mutations, and microsatellite instability (MSI).

MATERIALS AND METHODS

Tissue specimens

From 1982 to 1996, 22 patients with gallbladder carcinoma underwent a resection at Niigata University General Hospital and its affiliated institutions in Niigata Prefecture; the surgical specimens were collected and stored (Japanese cases). All the patients were Japanese. The Japanese cases in this study were identical with the Japanese cases in our previous study[14]. From 1982 to 2001, 20 patients with gallbladder carcinoma underwent a resection in hospitals in Budapest, Hungary; the surgical specimens were collected and stored (Hungarian cases) through the courtesy of a Hungarian oncologist (I.L.) and surgical pathologist (Z.S.). All the patients were residents in Budapest. Both the Japanese cases and the Hungarian cases (a total of 42 cases) formed the basis of this retrospective study. All of the patients gave informed consent for pathologically examining the specimens. No patient in this series was diagnosed with anomalous union of the pancreatic and biliary ducts (AUPBD), and had a family history suggestive of hereditary nonpolyposis colorectal cancer (HNPCC).

The surgical specimens were fixed in formalin and embedded in paraffin. One or two representative sections of each specimen were used for DNA analysis. Histopathological findings were described according to the tumor-node-metastasis (TNM) staging system[18].

DNA preparation

Five serial slices 10-μm thick were cut from each representative histologic section and deparaffinized. Cancer tissue in each slice was dissected under microscopic guidance as described previously[14]. Non-neoplastic tissue in each slice was used as a source of constitutional DNA. DNA was extracted using a DNA Isolator PS Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as described previously[14].

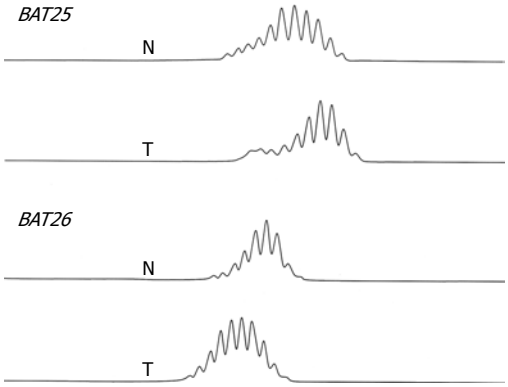

Figure 1 Fragment pattern of case No 1 (Table 1) showing microsatellite instability at two loci (*BAT25*, *BAT26*). N: Normal DNA; T: Tumor DNA.

Analysis of p53 mutations

p53 mutations at exons 5 to 8 were tested by nested polymerase chain reaction (PCR) and direct sequencing as described previously[14].

Analysis of K-ras mutations

K-*ras* mutations at codon 12 were tested by nested PCR, PCR-restriction fragment length polymorphism, and direct sequencing as described elsewhere[19].

Analysis of MSI

Fluorescent dye-labeled PCR amplification was performed using the five microsatellite markers (*BAT-25*, *BAT-26*, *D2S123*, *D5S346*, and *D17S250*) recommended by the National Cancer Institute workshop[20,21]. Fluorescent dye-labeled and unlabeled primers were obtained (Applied Biosystems Japan Ltd., Tokyo, Japan); the 5' oligonucleotide was end-labeled with 6FAM (*BAT-25*, *D2S123*), VIC (*BAT-26*, *D17S250*), or NED (*D5S346*) fluorescent dyes. Amplification by PCR was performed using the Temp Control System PC-700 (ASTEC Co., Ltd., Fukuoka, Japan) in 30-μL reaction volumes containing 100 ng of DNA, 0.75 U of AmpliTaq Gold (Applied Biosystems Japan Ltd.), and 10 pmol of each primer. The cycling profile was: denaturation at 95℃ for 2 min, annealing at 55℃ for 40 s and extension at 72℃ for 40 s followed by a 7-min final extension step. A 1.0-μL aliquot of each fluorescent dye-labeled PCR product was combined with 12 μL of formamide and 0.5 μL of the GENESCAN 350 [ROX] size standard (Applied Biosystems Japan Ltd.) and analyzed on an ABI PRISM 310 Genetic Analyzer using GeneScan Analysis Software (Applied Biosystems Japan Ltd.). MSI experiments were repeated twice to exclude the possibility of a false positive result due to PCR amplification artifacts in an independent PCR reaction. The sequencing results were verified by a second independently generated PCR product.

The presence of MSI was determined from the PCR amplifications of the five-microsatellite markers. Microsatellite instability was defined as the presence of novel peaks that were not found in non-neoplastic tissue (Figure 1). A tumor was defined as having high microsatellite instability (MSI-high) if more than one of

the five loci analyzed showed unequivocal instabilities; a tumor was defined as having low microsatellite instability (MSI-low) if only one locus showed instability; and a tumor was defined as microsatellite stable if no microsatellite instability was found. In this study, MSI-low tumors and microsatellite-stable tumors were categorized together into one group (MSI-low/none), as proposed by recent authors[16,22].

Statistical analysis

Fisher's exact test was used to compare the frequencies of genetic alterations between the two groups. All statistical evaluations were performed using the SPSS 11.5J software package (SPSS Japan Inc., Tokyo, Japan). All tests were two-sided and a P value < 0.05 was considered statistically significant.

RESULTS

p53 mutation

Mutations of *p53* at exons 5 to 8 were detected in 11 of 22 (50.0%) Japanese cases and 6 of 18 (33.3%) Hungarian cases ($P = 0.348$; Table 1). Among the 17 cases with *p53* mutations, transversion was found in 4 of 11 Japanese cases and 1 of 6 Hungarian cases ($P = 0.600$). Transition at CpG sites was found in none of 11 Japanese cases and 2 of 6 Hungarian cases; the difference was marginally significant ($P = 0.110$).

K-ras mutation

K-*ras* mutations at codon 12 were detected in none of 22 Japanese cases and 1 of 20 Hungarian cases ($P = 0.476$) (Table 1).

MSI

Eight of 19 (42.1%) Japanese cases were MSI-high, whereas only one of 15 (6.7%) Hungarian cases was MSI-high; the difference was statistically significant ($P = 0.047$; Table 1).

Association between p53 mutation and MSI in gallbladder carcinoma

Both *p53* mutation and MSI were analyzed successfully in 34 cases: 19 Japanese cases and 15 Hungarian cases (Table 1). When the cases were stratified into Japanese cases and Hungarian cases, *p53* mutations were not associated significantly with MSI in both groups. In all 34 cases, *p53* mutations were associated significantly with MSI (Table 2; $P = 0.033$).

DISCUSSION

Although earlier epidemiologic studies have suggested that the incidence of gallbladder carcinoma varies geographically or ethnically[9,10], there is a paucity of evidence regarding the geographic diversity of molecular changes associated with this tumor[7,8]. This prompted us to conduct the current study. In 1998, we reported that the *p53* mutational spectra of gallbladder carcinoma differed considerably between Japan and Chile, both of which were known as high-incidence countries[14]. The current study first demonstrates that the process of gallbladder carcinogenesis differs in terms of *p53* mutations and microsatellite instability between two distinct high-incidence areas: Japan and Hungary (a representative country of Eastern Europe).

Reported prevalences of *p53* mutation for gallbladder carcinoma ranged from 31% to 70%[14,23-26], suggesting that *p53* mutation plays an important role in the development of this tumor[8]. The prevalences of *p53* mutation in this series (50% in Japanese cases and 33% in Hungarian cases) are comparable with the reported figures. In various malignancies, endogenous carcinogenesis is characterized by transitions at CpG sites, whereas transversions imply the presence of an exogenous mutational process[27-29]. In our previous study, the prevalence of transitions at CpG sites was significantly higher in the Chilean cases than in the Japanese cases[14]. In the current study, there was a trend toward a higher prevalence of transitions at CpG sites in the Hungarian cases. Taken together, these observations suggest that an endogenous mutational process contributed considerably to carcinogenesis of the gallbladder in the Chilean and Hungarian cases. In contrast, considering that there were no transitions at CpG sites and four transversions in the Japanese cases, it appears that exogenous mutations often happen during the process of carcinogenesis in the Japanese cases. Therefore, geographic variation might exist in carcinogenesis of the gallbladder among three distinct areas.

Reported prevalences of K-*ras* mutation for gallbladder carcinoma without AUPBD ranged from 0% to 17%[16,30-33], suggesting that frequency of K-*ras* mutation is relatively low in gallbladder carcinoma without AUPBD. This is consistent with our results: K-*ras* mutation was found in only one of the Hungarian cases. Taken together, most gallbladder carcinoma appears to develop from a K-*ras*-independent pathway.

Microsatellite instability, which represents replication errors, results from DNA mismatches due to environmental or hereditary factors and leads to genomic instability[34,35]. Reported prevalences of MSI-high for sporadic gallbladder carcinoma range from 0% to 10%[15,16,36-38], whereas the prevalence was high (42.1%) in our cases. This suggests that environmental or hereditary factors contribute to carcinogenesis of the gallbladder in some of our cases. In the current study, the prevalence of MSI-high was significantly higher in the Japanese cases than in the Hungarian cases. Considering that there were no patients with HNPCC in this series, the above finding suggests that the high prevalence of MSI-high in the Japanese cases may be due to environmental factors, which remain unknown.

There are inverse relationships between *p53* mutations and MSI in colorectal cancer[39,40]. Regarding gallbladder carcinoma, earlier authors have failed to identify such relationships between *p53* mutations and MSI[16,38]. In this study, *p53* mutations were associated significantly with MSI in all cases. When the cases were stratified into Japanese cases and Hungarian cases, no such associations between *p53* mutation and MSI were found, probably due to the small sample size. Taken together, the above observations suggest that the pathway of carcinogenesis differs between the gallbladder and the colon and rectum.

The current study has limitations. Firstly, it was a

Table 1 Genetic alterations observed in gallbladder carcinomas from Japan and Hungary

Case No	Age (yr)	Sex	Histology[1]		*p53* mutation		K-*ras* mutation	Microsatellite instability
			Type	Grade	Exon/codon	Base change	Base change	
Japanese cases								
1	71	F	PAP	G1	None	-	None	BAT25, BAT26
2	74	F	AD	G1	Exon 5/codon 132	AAG to GAG	None	BAT25, D2S123
3	62	F	PAP	G1	None	-	None	BAT25
4	76	F	AD	G1	Exon 6/codon 193	CAT to AAT	None	BAT25, BAT26
5	78	F	AD	G2	Exon 5/codon 140	ACC to ATC	None	ND
					Exon 5/codon 166	TCA to ACA		
6	70	F	AD	G1	Exon 8/codon 276	GCC to CCC	None	None
7	71	F	AD	G1	None	-	None	BAT26, D2S123
8	79	F	AD	G1	None	-	None	None
9	66	F	PAP	G1	None	-	None	None
10	60	F	PAP	G1	None	-	None	None
11	80	M	PAP	G1	None	-	None	BAT26
12	63	M	PAP	G1	Exon 8/codon 294	GAG to GAA	None	BAT25, BAT26
13	49	F	PAP	G1	None	-	None	D2S123
14	71	M	AD	G2	Exon 8/codon 280	AGA to AAA	None	BAT25, D2S123
15	70	M	AD	G1	Exon 7/codon 238	TGT to CGT	None	ND
16	53	M	AD	G1	None	-	None	None
17	79	F	AD	G3	Exon 8/codon 271	GAG to AAG	None	None
18	62	M	AD	G3	Exon 6/codon 205	TAT to TGT	None	None
19	61	M	AD	G1	None	-	None	D17S250, D2S123
20	69	M	AD	G2	Exon 7/codon 231	ACC to ATC	None	BAT25, D17S250
21	58	F	AD	G2	Exon 5/codon 160	ATG to GTG	None	ND
					Exon 6/codon 220	TAT to AAT		
22	76	F	AD	G3	None	-	None	None
Hungarian cases								
23	71	M	AD	G2	Exon 7/codon 249	AGG to ACG	None	BAT26
24	69	F	AD	G3	Exon 6/codon 213	CAG to CGG	None	None
25	69	F	AD	G2	None	-	None	None
26	76	F	AD	G2	None	-	None	None
27	61	F	AD	G3	None	-	None	None
28	58	F	AD	G3	None	-	None	BAT25
29	69	F	AD	G3	None	-	None	None
30	75	F	PAP	G1	None	-	None	None
31	66	F	AD	G3	None	-	None	None
32	65	F	AD	G1	Exon 6/codon 219	CCC to TCC	None	ND
33	61	F	AD	G3	ND	-	None	ND
34	Unknown	F	PAP	G1	Exon 5/codon 138	GCC to GCT	None	BAT25, D17S250
					Exon 5/codon 167	CAG to CAA		
					Exon 5/codon 170	ACG to ACA		
35	Unknown	F	PAP	G1	Exon 5/codon 158	CGC to TGC	GGT to AGT	ND
					Exon 6/codon 223	CCT to TCT		
36	Unknown	F	AD	G3	Exon 6/codon 210	AAC to AAT	None	ND
					Exon 7/codon 228	GAC to AAC		
37	Unknown	F	AD	G2	ND	-	None	ND
38	53	F	AD	G3	None	-	None	None
39	72	Unknown	AD	G1	None	-	None	None
40	69	M	AD	G1	None	-	None	BAT26
41	57	F	AD	G1	None	-	None	None
42	74	F	AD	G1	None	-	None	BAT25

[1]According to the tumor-node-metastasis (TNM) staging system[18]. PAP: Papillary carcinoma; AD: Adenocarcinoma; ND: Not detected; G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated.

Table 2 Association between *p53* mutation and MSI in gallbladder carcinoma

		MSI-high	MSI-low/none	*P*
p53 mutation	Present	6	5	0.033
	Absent	3	20	

MSI: Microsatellite instability.

retrospective analysis of a small number of patients. Secondly, DNA preparation from paraffin-embedded tissue sections was unsuccessful in some patients. Thirdly, only limited clinical information was available in the Hungarian cases to protect the patients' privacy. To our knowledge, however, this study demonstrates more clearly the geographic diversity of gallbladder carcinogenesis than earlier reports[15-17].

In conclusion, it appears that the *p53* mutations and MSI differ in patients with gallbladder carcinoma between two distinct high-incidence areas. Exogenous mutations

and unknown environmental factors may play roles in gallbladder carcinogenesis in the Japanese cases, whereas the Hungarian cases are characterized by an endogenous mutational process. Thus, geographic variation might exist in the process of gallbladder carcinogenesis.

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COMMENTS

Background

Although considerable progress has been made regarding the molecular pathogenesis of human neoplasms, there is only limited information about the genetic changes involved in gallbladder carcinogenesis. Gallbladder carcinoma shows striking geographic and ethnic variation, however there is a paucity of evidence regarding the geographic diversity of molecular changes associated with this tumor.

Research frontiers

In 1998, we reported that the *p53* mutational spectra of gallbladder carcinoma differed considerably between Japan and Chile, both of which were known as high-incidence countries. Only a few other investigators also have reported such geographic and ethnic differences in genetic changes of this tumor. The aim of this study was to disclose geographic differences in genetic changes involved in gallbladder carcinogenesis by comparing gallbladder carcinomas from two distinct high-incidence areas, Japan and Hungary.

Innovations and breakthroughs

The current study first demonstrates that the *p53* mutations and MSI differ in patients with gallbladder carcinoma between two distinct high-incidence areas: Japan and Hungary. Exogenous mutations and unknown environmental factors may play roles in gallbladder carcinogenesis in the Japanese cases, whereas the Hungarian cases are characterized by an endogenous mutational process. Thus, geographic variation might exist in the process of gallbladder carcinogenesis.

Applications

Gallbladder carcinoma is a highly lethal disease with a poor prognosis. Therefore, it would be very beneficial to identify the molecular mechanism responsible for this condition. Advances in the understanding of the genetic changes of this tumor will help in understanding the pathogenesis of this miserable disease.

Terminology

Microsatellite instability (MSI) is caused by a failure of the DNA mismatch repair system to repair errors that occur during the replication of DNA and is characterized by the accelerated accumulation of single nucleotide mutations and alterations in the length of simple, repetitive microsatellite sequences that occur ubiquitously throughout the genome. MSI is seen in most HNPCC tumors and proportion of nonhereditary colorectal tumors. The presence of MSI in tumor tissue is associated with certain unique clinical and pathological characteristics.

Peer review

This study aimed to determine the difference of some genetic changes involved in gallbladder carcinogenesis in high-prevalence areas of Japan and Hungary. This is a well written paper and has a nice finding. While it is simply an observational study, I think it is an important observation.

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