Multiple K-*ras* Mutations in Hyperplasia and Carcinoma in Cases of Human Pancreatic Carcinoma

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Mucous cell hyperplasia (MCH) has been considered an important precursor of pancreatic ductal carcinoma based on histological and molecular research, although various K-*ras* mutations rates are seen among cases with pancreatic carcinoma, chronic pancreatitis and normal pancreas, with a wide range of histological characters. To investigate the premalignant potential of MCH and the multicentricity of pancreatic carcinoma, we analyzed K-*ras* mutation at codon 12 in carcinoma foci of 82 cases of surgically-resected pancreatic carcinoma [67 solid-type carcinomas (SCs) and 15 ductectatic-type carcinomas (DCs)], as well as in both MCH and carcinoma foci in 42 cases (30 SCs and 12 DCs), using an enriched polymerase chain reaction (PCR)-enzyme linked mini-sequence assay (ELMA). K-*ras* mutation was recognized in 85% (57/67) of SCs and 73% (11/15) of DCs, and multiple K-*ras* mutations in 12% (8/67) of SCs and in 20% (3/15) of DCs. Multiple K-*ras* mutations were also recognized in MCHs in 47% (14/30) of SCs and in 42% (5/12) of DCs. Moreover, the same sequence at K-*ras* codon 12 in MCH and carcinoma was identified in 76% (32/42) of carcinoma cases and it was more frequently recognized in hyperplasias with histological atypia (51%, 37 of 72 foci) than those without atypia (24%, 16 of 68 foci) (*P*<0.0007). These results further support the idea of multicentric carcinogenesis and premalignant potential of atypical hyperplasia in the human pancreas, although about half of the hyperplasias around carcinomas were not thought to be direct precursors.

Key words: Pancreas — K-*ras* — Multicentricity — Carcinogenesis — Human

Histological and molecular analytical aspects of carcinogenesis are of clinical importance to detect early cancers. Mucous cell hyperplasia (MCH), especially atypical hyperplasia, of the pancreatic duct has been thought to be a significant precursor of pancreatic ductal carcinoma, because it is frequently observed as physically continuous with cancer tissue[1–3] and the mucus characteristics of MCH are similar to those of carcinomas.[3] It has also been reported that a high incidence of K-*ras* mutation at codon 12 was detected in MCH,[4–7] as well as in pancreatic ductal carcinoma,[7–14] and the same sequence of K-*ras* mutations in MCH and carcinoma was frequently (8 of 10 cases) recognized in a single pancreas.[6] Therefore MCH with K-*ras* mutation might represent a high-risk precursor of invasive carcinoma. However, it is still unclear if all K-*ras* mutant hyperplasias surrounding carcinoma are precancerous.

Multicentricity of pancreatic ductal carcinoma has been reported in 16–38% of patients by histological examination.[15–19] It is also supported by previous reports showing multiple K-*ras* mutations in 3–17% of pancreatic cancers.[11–14] However, few studies have analyzed sequences of K-*ras* codon 12 in both MCH and carcinoma in the same pancreas with large numbers of cases and foci. This time, to investigate pancreatic multicentric carcinogenesis, we analyzed K-*ras* mutation in large numbers of MCH and carcinoma foci in cases with pancreatic cancer, using a highly sensitive and non-isotopic assay for detecting K-*ras* codon 12 mutation.

MATERIALS AND METHODS

Human pancreatic specimens Specimens were selected from consecutively recorded cases in our archives at the First Department of Pathology, Niigata University School of Medicine; 67 surgical cases of solid-type carcinoma (SC)[7, 20, 21] (equivalent to "ductal adenocarcinoma" in the WHO classification[22] and IPCSG[23]) (34 men and 33 women, mean age±SD: 66.1±8.1, range 44–79 years old) and 15 surgical cases of ductectatic-type carcinoma (DC)[7, 20] [equivalent to "intraductal (papillary-mucinous) carcinoma" and "mucinous cystadenocarcinoma"] (12 men and 3 women, mean age±SD: 68.8±8.1, range 48–77 years old). All specimens were fixed in 10% formalin,

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serially sliced into 3 to 4 mm sections, and embedded in paraffin. The clearest sections (1–4 sections per case) for analyzing target foci were serially sliced into specimens for hematoxylin-eosin (HE) staining and DNA extraction.
Classification of pancreatic ductal epithelia In HE specimens, pancreatic ductal epithelia were divided into the following five groups: ordinary epithelium, MCH including nonpapillary type [including "adenomatoid ductal hyperplasia," "non-papillary epithelial hypertrophy" and/or "mucinous cell hypertrophy"][22, 23] and papillary type (Fig. 1, C–G), and carcinoma including intraductal (Fig. 1H) and invasive carcinoma (Fig. 1B). No adenoma com-
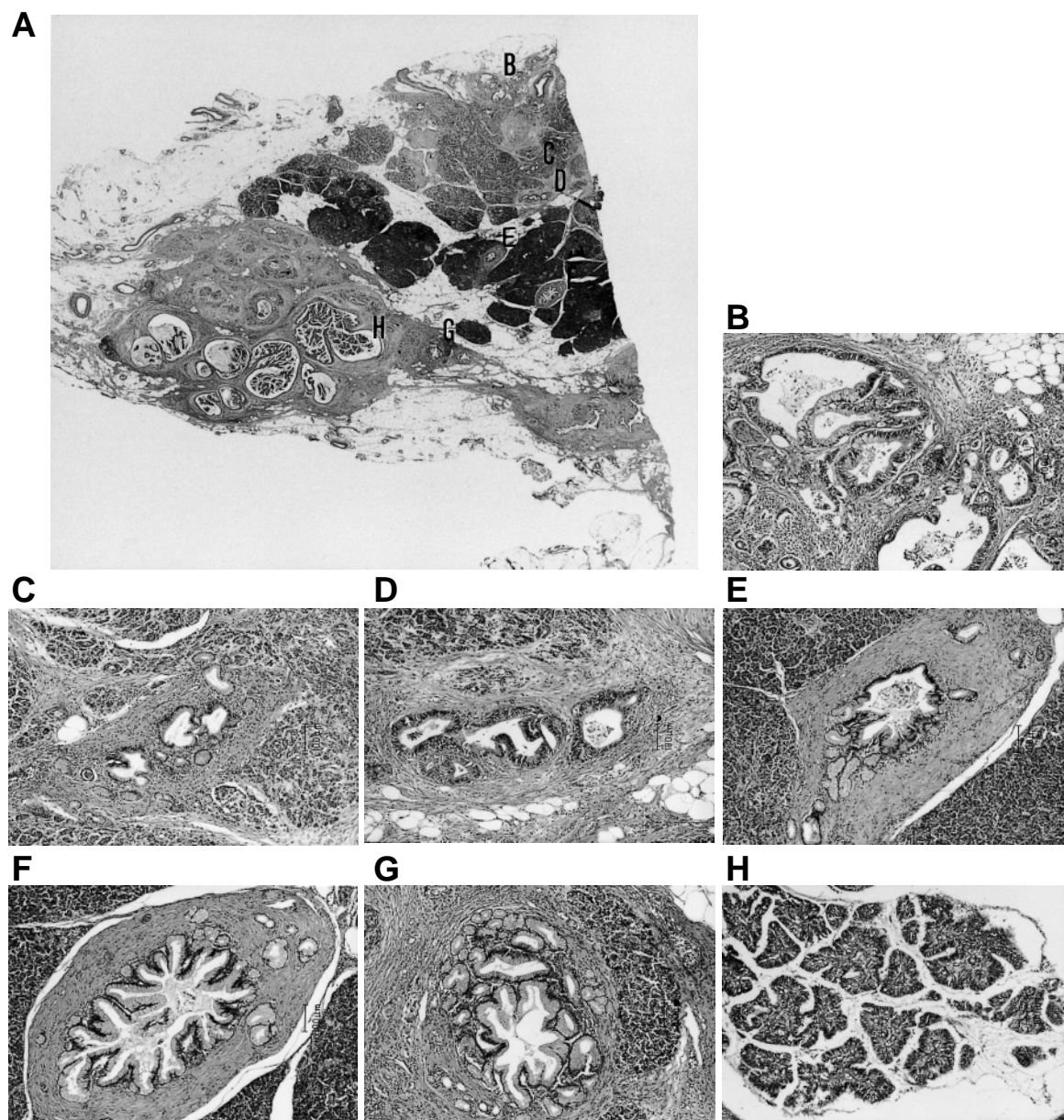

Fig. 1. Low-power view of ductectatic-type carcinoma (DC) (case No. 42) accompanied with multiple hyperplastic foci (A: HE). Focus of invasive carcinoma having GAT-type mutation at K-*ras* codon 12 (B: ×25). Papillary type MCH having GAT- and GTT-type mutation (C: ×25). Papillary-type MCH with atypia having GAT-type mutation (D: ×25). Papillary-type MCH having GAT-type mutation (E: ×25). Papillary-type MCH having GTT- and GCT-type mutation (F: ×25). Papillary-type MCH having GTT-type mutation (G: ×25). Intraductal papillary carcinoma component having GAT-type carcinoma (H: ×25).

ponent was examined. Histological atypia of MCHs (Fig. 1D) was examined, referring to previous reports,[1–3, 22–24] with the following histological criteria: high columnar epithelia, forming flat or papillary structures with fibrous cores (sometimes not seen in diagonally sliced specimen), sometimes crowded, with (partial or) no loss of polarity, basically basally located and round or irregular-shaped enlarged nuclei, with either vesicular or dense hyperchromatic but not large dot-type chromatin pattern, with sporadically or focally aggregated basophilic nucleoli.

Sampling of nonneoplastic and neoplastic pancreatic ductal epithelia analyzed for K-*ras* mutation A hundred and forty MCH foci [86 foci (range, 1–14; mean±SD, 2.9±2.5 foci per case) from 30 SCs and 54 foci (range, 1–12; mean±SD, 4.5±4.0 foci per case) from 12 DCs] were sampled from pancreata with primary carcinomas. Most (more than 80%) of the MCH foci were located within about 10 mm from the edge of the carcinoma lesion. Besides, 45 carcinoma foci (15 intraductal and 30 invasive foci; range, 1–4; mean±SD, 1.5±0.8 foci per case) were sampled from 30 SCs, and 14 carcinoma foci (11 intraductal and 3 invasive foci; range, 1–2; mean±SD, 1.2±0.4 foci per case) from 12 DCs. Every target focus was checked for cytological features on two HE specimens, on either side of the specimen for DNA extraction. To avoid DNA contamination between MCH and carcinoma, we did not select small areas containing MCH-carcinoma transition, and always dissected MCH before dissecting the carcinoma.

DNA extraction The target focus, which was composed of about 50–2000 cells, was dissected with a disposable fine needle under microscopic observation. Details of DNA extraction were described in the previous study.[7]

Extracted DNA was dissolved in 50 μl of distilled water (Kanto Chemical, Tokyo) and kept at −80°C.

Analysis of K-*ras* mutation at codon 12 Point mutation of K-*ras* codon 12 was analyzed by enriched polymerase chain reaction (PCR)-enzyme linked mini-sequence assay (ELMA) (Sumitomo Metal Industry, Inc., Tokyo) (Fig. 2). This method made it possible to identify highly sensitively and non-isotopically the sequences at K-*ras* codon 12 in one working day.

The oligonucleotide primers were as follows: upstream for the first and second PCR; 5′-TAAACTTGTGGTAGT-TGGAACT-3′, downstream for the first PCR; 5′-GTTG-GATCATATTCGTACAC-3′, and downstream for the
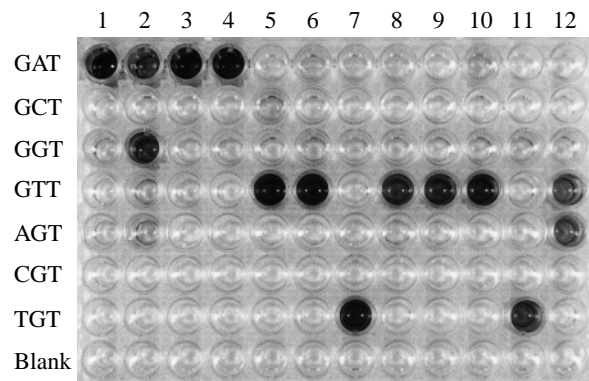

Fig. 2. Enriched polymerase chain reaction (PCR)-enzyme linked mini-sequence assay (ELMA), showing K-*ras* codon 12 mutation in carcinoma and hyperplasia foci: GAT-type mutation in lanes 1–4 (wild-type DNA is also positive in lane 2), GTT-type mutation in lanes 5, 6, 8–10, TGT-type mutation in lanes 7 and 11, and GTT- and AGT-type mutations in lane 12.

Table I. K-*ras* Codon 12 Mutation of Pancreatic Carcinomas

Sequence of K-*ras* codon12	Type of carcinoma	
	Solid-type carcinoma 67 cases	Ductectatic-type carcinoma 15 cases
Single mutation	49 cases (73%)	8 cases (53%)
GTT (Val)	24 (36%)	5 (33%)
GAT (Asp)	19 (28%)	2 (13%)
CGT (Arg)	5 (7%)	0
AGT (Ser)	1 (1%)	0
TGT (Cys)	0	1 (7%)
Multiple mutations	8 cases (12%)	3 cases (20%)
GTT (Val) + GAT (Asp)	5 (7%)	1 (7%)
GAT (Asp) + CGT (Arg)	1 (1%)	1 (7%)
GAT (Asp) + AGT (Ser)	2 (3%)	0
GAT (Asp) + GCT (Ala)	0	1 (7%)
Wild-type		
GGT (Gly)	10 cases (15%)	4 cases (27%)

Table II. K-*ras* Codon 12 Mutation of Mucous Cell Hyperplasias and Carcinomas in Cases of Pancreatic Carcinoma

Case No.	Type of carcinoma[a]	Age/sex	ts[b]	Sequence of K-*ras* codon 12		
				Mucous cell hyperplasia	Carcinoma	
					Intraductal	Invasive
1	SC	61/F	1b	GTT GAT GAT WT[c] WT	GAT	GTT GTT
2	SC	63/M	1b	GAT CGT	GAT GAT	
3	SC	73/M	1b	GAT GAT + GTT WT	GAT	GAT
4	SC	67/M	1b	WT WT WT TGT GAT		WT
5	SC	63/F	1b	GTT TGT		CGT
6	SC	74/F	1b	WT GAT GTT		WT
7	SC	63/M	2a	WT WT		GAT + GTT
8	SC	51/M	2a	GAT GAT	GAT	
9	SC	76/F	2a	WT		GAT
10	SC	59/F	2a	GTT	GAT	
11	SC	68/F	2a	GTT WT		GTT
12	SC	76/M	2a	GTT GAT GAT GAT TGT		GTT
13	SC	60/F	2a	GTT GTT GTT GTT GAT GAT		GTT
14	SC	63/F	2b	GTT CGT WT		GTT
15	SC	73/M	2b	CGT + GTT		GAT
16	SC	70/F	2b	GTT + GAT CGT CGT		GTT
17	SC	66/F	2b	GAT	GAT	GAT
18	SC	70/F	2b	GAT		GAT
19	SC	57/M	2b	GAT GAT		GAT
20	SC	64/M	3	GTT		GAT
21	SC	70/F	3	CGT CGT CGT GTT GTT GTT GTT GTT GTT GTT GTT GTT GAT GAT	CGT	CGT CGT
22	SC	62/M	3	GTT	GTT	GTT
23	SC	75/M	3	GTT GAT CGT		GTT

Table II. (Continued)

Case No.	Type of carcinoma[a]	Age/sex	ts[b]	Sequence of K-*ras* codon 12		
				Mucous cell hyperplasia	Carcinoma Intraductal	Invasive
24	SC	72/F	3	WT WT	GTT	GTT
25	SC	67/F	3	WT CGT		WT WT
26	SC	72/F	3	GAT		GTT
27	SC	66/M	3	GTT GTT WT	GTT GTT GTT	GTT
28	SC	61/M	3	GAT GAT	GAT	GAT
29	SC	60/M	3	GTT GTT GTT GAT WT		GTT
30	SC	56/M	4	GAT WT	GAT	GAT
31	DC	70/M	1a	WT	WT	
32	DC	72/M	1a	GTT GTT GTT GTT GTT / GTT GTT GTT GTT GTT / GTT GTT GTT WT	GTT GTT	
33	DC	75/M	1a	WT GTT	WT	
34	DC	72/M	1b	WT CGT		WT
35	DC	60/F	2a	GTT GTT GTT GTT CGT / WT WT	GTT	
36	DC	77/M	2b	GTT GTT GTT GTT / GTT GTT GAT WT	GTT	
37	DC	72/M	2b	GAT + GTT GTT WT	GAT + GTT	
38	DC	63/M	3	GTT WT	GTT	
39	DC	58/M	3	GTT GTT + AGT CGT TGT	GAT	
40	DC	74/M	4	GAT	TGT	
41	DC	34/F	4	GTT GTT		GTT
42	DC	74/F	4	GAT GAT GAT + GTT GTT / GTT GTT GTT GTT + GCT	GAT	GAT

a) SC: solid-type carcinoma, DC: ductectatic-type carcinoma.
b) ts: tumor size (ts1a≤1 cm, 1 cm<ts1b≤2 cm, 2 cm<ts2a≤3 cm, 3 cm<ts2b≤4 cm, 4 cm<ts3≤6 cm, 6 cm<ts4). □ one focus, ■ MCH with atypia.
c) WT: wild type at K-*ras* codon 12 (GGT).

second PCR; 5′-CAAATGATCTGAATTAGCTG-3′. The first PCR was carried out with 25 μl of reaction mixture containing 1 μl of template DNA, 100 μM deoxyribonucleotide triphosphates (dNTPs) containing dATP, dCTP, dGTP and dTTP, 1.5 mM MgCl$_2$, 1 μM each primers, 0.625 U of *Taq* DNA polymerase (Perkin Elmer, Norwalk, CT) and 1× PCR buffer [containing 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl and 0.001% (w/v) gelatin] in a thermal cycler (Perkin Elmer PJ-2000). For the first PCR, after an initial denaturation at 95°C for 2 min, 25 cycles of denaturation at 95°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 40 s were performed. One microliter of 10-fold dilution of the first PCR product was digested with 2.5 U of *Bsr*I restriction enzyme (New England Biolabs, Beverly, MA) and 3.5 μl of enzyme-reaction buffer [100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl$_2$ and 1 mM dithiothreitol (DTT)] at 65°C for at least 15 h. Using this digested solution as a template DNA, the second PCR was performed in 50 μl of reaction mixture for 40 cycles under the same conditions and with the same initial denaturation as in the first PCR. The second PCR product was mixed with 50 μl of denaturing solution and 10 μl of the denatured PCR product was hybridized with probes in a 96-microwell plate, on which oligonucleotide probes for detecting the K-*ras* codon 12 wild-type (GGT) and six mutants (GAT, GCT, GTT, AGT, CGT and TGT) DNA were immobilized, at 55°C for 30 min. After washing out of the hybridized product, 100 μl of biotinated A and 0.01 U of *Taq* DNA polymerase were added and incubation was continued at 55°C for 30 min. To develop color, 100 μl of avidin-horseradish peroxidase conjugate was added and the mixture was kept at room temperature for 30 min. After a washing step, 100 μl of tetramethylbenzidine (TMB) substrate was added and the plates were kept in the dark at room temperature for 20 min. Finally, 100 μl of stop solution was added and the light absorbance

of each sample was measured by spectrophotometry (Multiskan Multisoft, Labsystems, Tokyo) with a 450 nm filter wavelength.

In preliminary studies, the reliability of this method was ascertained by repeated analysis using DNAs extracted from cell lines, the K-*ras* sequences of which were already known. In addition, using various mixtures of several kinds of plasmid DNAs, the sensitivity of this method was determined to be 0.2–2% mutant DNA concentration.

Statistical analysis Differences in age were evaluated by use of the Mann Whitney *U*-test. Differences in gender, frequency of K-*ras* mutation, and MCH with atypia were evaluated with the χ^2 test (two-sided). A probability value of less than 0.05 was considered statistically significant.

RESULTS

K-*ras* mutation in pancreatic ductal carcinomas Of 67 SCs, K-*ras* mutation was recognized in 57 (85%) cases, among which a single mutation was detected in 49 (73%) cases [according to the mutation type, 24 (36%) GTTs (K-*ras* codon 12: GGT→GTT), 19 (28%) GATs, 5 (7%) CGTs and 1 (1%) AGT] and multiple mutations in 8 (12%) [5 (7%) GTTs and GAT, 2 (3%) GAT and AGT, and 1 (1%) GAT and CGT]. Of 15 DCs, K-*ras* mutation was recognized in 11 (73%) cases, among which a single mutation was detected in 8 (53%) cases [5 (33%) GTTs, 2 (13%) GATs, and 1 (7%) TGT], and multiple mutations in 3 (20%) cases [1 (7%) GTT and GAT, 1 (7%) GAT and CGT, and 1 (7%) GAT and GCT] (Table I).

Relation of K-*ras* mutation in pancreatic ductal hyperplasia and carcinoma As shown in Table II, 30 cases of SC and 12 cases of DC, among which 27 (90%) cases of SC and 9 (75%) cases of DC had K-*ras* mutation in carcinoma foci, were chosen for analysis of K-*ras* mutation in MCH foci. K-*ras* mutant MCH was recognized in 27 of 30 (90%) cases and 68 of 86 (79%) foci in SC cases, and 11 of 12 (92%) cases and 45 of 54 (83%) foci in DCs. Mutation types of MCH in pancreatic carcinoma cases (including SCs and DCs) were as follows: GTT in 26 (62%) cases and 68 (49%) foci, GAT in 22 (52%) cases and 33 (24%) foci, CGT in 10 (24%) cases and 13 (9%) foci, TGT in 4 (10%) cases and 4 foci (3%), AGT in 1 (2%) case and 1 (1%) focus, and GCT in 1 (2%) case and 1 (1%) focus. Multiple K-*ras* mutations in MCH foci (Fig. 1, Table II) were recognized in 14 of 30 (47%) SCs and 5 of 12 (42%) DCs.

MCH having the same mutation sequence at K-*ras* codon 12 as carcinoma was recognized in 32 of 42 pancreatic carcinoma cases [19 of 27 (70%) K-*ras* mutant SCs, all of 3 K-*ras* wild-type SCs, 7 of 9 (78%) K-*ras* mutant DCs and all of 3 K-*ras* wild-type DCs]. Furthermore, the same K-*ras* sequence as in carcinoma was identified in 72 of 140 (51%) MCH foci, increasing from 40% (35/87) of

Table III. Correlation between Histological Atypia and K-*ras* Mutation of MCH

	Incidence of MCH with the same K-*ras* codon 12 sequence as carcinoma (No. of foci)	
	MCHs without atypia: %	MCHs with atypia: %
K-*ras* mutant MCHs	45 (29/65)	73 (35/48)
	P=0.003	
K-*ras* wild-type MCHs	27 (6/22)	40 (2/5)
Total	40 (35/87)	70 (37/53)
	P=0.0007	

MCH: mucous cell hyperplasia.

MCH foci without atypia to 70% (37/53) of those with atypia (*P*=0.0007) (Table III).

DISCUSSION

Our previous study,[7] which did not analyze the mutational type, showed increasing incidence of K-*ras* mutation at codon 12 from non-papillary hyperplasias to papillary ones, which supports the hypothesis of a sequence from non-papillary through papillary hyperplasias to carcinoma as the main route of pancreatic carcinogenesis. In the current study, to further test the hypothesis, we analyzed the sequence of K-*ras* codon 12 in pancreatic epithelial foci with large numbers of samples. As a result, the same type of K-*ras* mutation as in the carcinoma was frequently recognized in MCHs with increasing incidence from foci without atypia to those with atypia, which is compatible with the report by Moskaluk *et al.*[6] Yanagisawa *et al.*[4] have reported frequent K-*ras* mutation in hyperplasia without nuclear atypia (10 of 16 foci, 63%) in cases of chronic pancreatitis. Hence, K-*ras* activation is thought to occur at a very early stage of pancreatic carcinogenesis and may not directly indicate high premalignant potential. From our data, atypical hyperplasias (not all K-*ras* mutant hyperplasias) are thought to be direct precursors of pancreatic carcinomas.

In our current study, we also detected multiple K-*ras* mutations in hyperplasias (42–47% of cases) as well as in carcinoma (12–20% of cases). Previous papers have reported multiple K-*ras* mutations in 3–17% of pancreatic carcinomas[11–14] and histological multicentricity in 16–38%.[15–19] In our data, about half (68/140) of MCHs surrounding carcinoma were different from the carcinoma in terms of K-*ras* sequence. Of these 68 MCHs, 49 (72%) foci were K-*ras* mutants. In previous reports, K-*ras* mutation rates of hyperplastic foci in normal pancreas ranged from 18% (9/51)[7] to 24% (19/79).[5] Therefore, it is possible that K-*ras* mutant hyperplasia is more likely to develop in pancreata having carcinoma than in the normal pancreas. Klöppel *et al.*[24] reported that a high incidence of papillary hyperplasias was recognized in mild to moderate obstructive pancreatitis caused by carcinoma invasion in the head of the pancreas. Brentnall *et al.*[25] reported that patients with pancreatic cancer or pancreatitis, in whom the pancreatic juice was positive for K-*ras* mutation, also

showed microsatellite instability. Furthermore, the amount of reactive oxygen species, which are thought to induce DNA damage, is high in pancreatitis,[26, 27] and such mutagenic stimuli may increase more in a pancreas with cancer. From the results of Furuya *et al.*,[28] it is not certain if carcinoma develops from K-*ras* mutant hyperplasia, although that was the conclusion drawn by Ochi *et al.*[29] based on the results of follow-up of a case of chronic pancreatitis in which the pancreatic juice harbored K-*ras* mutations. Similarly, it is unknown if a second cancer develops from K-*ras* mutant hyperplasia surrounding an existing cancer within the patient's life span, though the presence of multiple K-*ras* mutations in carcinoma and hyperplasia foci suggests that this may be possible.

The proportions of mutant sequences at K-*ras* codon 12 in carcinoma lesion were compatible with previous reports and most were GTT, GAT or CGT (Table I). Interestingly, the K-*ras* mutation type in MCH foci was almost the same as in carcinoma foci (Table II), which also supports the hyperplasia-carcinoma sequence in human pancreas. Tada *et al.*[5] reported that K-*ras* mutation types in carcinoma foci were GAT (53%), GTT (33%) and CGT (14%), in contrast to the dominance of TGT (37%) and AGT (16%) type mutation in MCHs in normal pancreas. Although we did not analyze MCHs in normal pancreata in the current study, at least hyperplasias having K-*ras* codon 12 mutation of GTT, GAT or CGT type are thought to have some degree of premalignant potential.

Our results support the hypothesis of pancreatic carcinogenesis via hyperplasia, though further investigations on mutagenic stimuli and molecular alterations in atypical hyperplasias and carcinomas are needed to detect early cancers.

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