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 mutation 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 tissue1–3 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, 13) 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 classification22) 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-

![Figure 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).](image-url)
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ponent was examined. Histological atypia of MCHs (Fig. 1D) was examined, referring to previous reports,\(^1\) 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′-TAACTTGTTGATGTGGAACT-3′, downstream for the first PCR; 5′-GTTGGATCATATTCTGAC3′, 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-types mutations in lane 12.](image)

| Table 1. K-ras Codon 12 Mutation of Pancreatic Carcinomas |
|-----------------|---------------------------------|---------------------------------|
| **Sequence of K-ras codon12** | **Type of carcinoma** | **Solid-type carcinoma** | **Ductectatic-type carcinoma** |
| Single mutation | | 67 cases | 15 cases |
| GTT (Val) | 49 cases (73%) | 8 cases (53%) |
| GAT (Asp) | 24 (36%) | 5 (33%) |
| CGT (Arg) | 19 (28%) | 2 (13%) |
| AGT (Ser) | 5 (7%) | 0 |
| TGT (Cys) | 1 (1%) | 0 |
| 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 | | 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 | Age/sex | t sub 3 | Mucous cell hyperplasia | Carcinoma |
|----------|-------------------|---------|---------|------------------------|-----------|
|          |                   |         |         |                        | Intraductal | Invasive |
| 1        | SC                | 61/F    | 1b      | GTT GAT GAT 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 + GAT |          |
| 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 GAT     | GTT       |          |
|          |                   |         |         |                        |           |          |
| 14       | SC                | 63/F    | 2b      | GTT CGT WT           |           |          |
| 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      |
| 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  | CGT       | CGT CGT  |
|          |                   |         |         | GTT GTT GTT GTT     |           |          |
|          |                   |         |         | GTT GTT GAT GAT     |           |          |
| 22       | SC                | 62/M    | 3       | GTT                 |           | GTT      |
| 23       | SC                | 75/M    | 3       | GTT GAT CGT         |           | GTT      |
Table II. (Continued)

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

c) SC: solid-type carcinoma, DC: ductectatic-type carcinoma.

<sup>a</sup> SC: solid-type carcinoma, DC: ductectatic-type carcinoma.

<sup>b</sup> 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.

<sup>c</sup> WT: wild type at K-ras codon 12 (GGT).
second PCR: 5′-CAATGTTCATCTGAATTAGCTG-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 MgCl₂, 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 restriction enzyme (New England Biolabs, Beverly, MA) and 3.5 µl of enzyme-reaction buffer [100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 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 mutation in pancreatic ductal carcinomas 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 χ² 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, 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 |
|---------------------------------|----------------|----------------|
|                                 | MCHs without  | MCHs with       |
|                                 | atypia: %      | 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.6) 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 carcinomas11–14) 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.20) 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.21) 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.20) it is not certain if carcinoma develops from K-ras mutant hyperplasia, although that was the conclusion drawn by Ochi et al.28) 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.29) 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 pancrea 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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