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Frequent Glycine-to-Aspartic Acid Mutations at Codon 12 of c-Ki-*ras* Gene in Human Pancreatic Cancer in Japanese

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Point mutations at codons 12 and 13 of c-Ki-*ras* gene were analyzed in human pancreatic cancer. DNAs obtained from sample tissues were amplified by means of polymerase chain reaction and were analyzed by dot blot hybridization assays with oligonucleotide probes appropriate for detecting mutations at these codons. Out of 38 evaluated cases, point mutations at codon 12 were found in 35 cases; these mutations resulted in changes of the coded amino acid to aspartic acid in 24 cases, to valine in 9 cases, to arginine in 2 cases and to cysteine in one case. In one case, a glycine-to-aspartic acid mutation was found at codon 13. In two cases, two distinct mutations were simultaneously present. The frequency pattern of mutations at codon 12 was somewhat different from those given in two previous reports on the similar analysis of pancreatic cancers in European countries. This may indicate the presence of possible genetic or non-genetic factors in determining preferential mutational patterns at these particular codons.

Key words: c-Ki-*ras* — Point mutation — Polymerase chain reaction — Pancreatic cancer

The activation of c-*ras* genes has been of considerable interest in the analysis of human oncogenesis because of its occurrence in a wide variety of human cancers.[1,2] Sequence analyses of c-*ras* genes which showed transforming activity in either *in vitro* or *in vivo* assays have consistently demonstrated the presence of point mutations in c-Ki-*ras*, c-Ha-*ras* and c-N-*ras* genes. These point mutations in c-*ras* genes have been confined, so far, to codons 12, 13 and 61 in human cancers.[3-5]

Recently, Almoguera *et al.*[6] reported a surprisingly high frequency of c-*ras* point mutations, in 21 out of 22 cases of human pancreatic cancer. All of the mutations were confined to codon 12 of c-Ki-*ras* gene. Since they utilized the RNAase A mismatch cleavage procedure, they were unable to determine the precise mutation sites. Subsequently Smit *et al.*[7] and Grünewald *et al.*[8] reported c-Ki-*ras* mutations in human pancreatic cancers by utilizing polymerase chain reactions (PCR) and hybridization assays with oligonucleotide probes. Both groups also found a high frequency of c-Ki-*ras* mutations at codon 12. According to their reports, however, the frequencies of mutation patterns at this particular codon were not necessarily similar. This raises the possibility of various genetic or non-genetic factors in oncogenic processing involving c-*ras* mutations and thus demands broader analyses of individual types of cancer.

We therefore analyzed 38 evaluated cases of pancreatic cancer in terms of c-Ki-*ras* point mutations. Although we again found a high incidence of c-Ki-*ras* mutations at codon 12, in 35 out of 38 cases, the frequency pattern of mutations at codon 12 was quite different from those of two previous reports.

The relevance of smoking in determining preference of mutations is also discussed.

MATERIALS AND METHODS

Tumor specimens Frozen specimens and 10% formalin-fixed and paraffin-embedded specimens of pancreatic cancer were obtained from the Second Department of Surgery, Nagasaki University School of Medicine between 1978 and 1989. The patients were 58% female, 42% male, and the age at diagnosis ranged from 47 to 82 years (median 61.6 years). Histological classification and stage of pancreatic cancer were determined according to the General Rules for Cancer of the Pancreas by the Japan Pancreas Society. These data and history of cigarette smoking for individual patients are all listed in Table I.

Preparation of cellular DNA Cellular DNA was prepared from frozen tissues by the method of Blin and Stafford[9] and also from the paraffin-embedded sections according to the method of Shibata *et al.*[10] for PCR-mediated amplification.

Synthetic oligonucleotides The oligonucleotides were synthesized by a DNA synthesizer (model 380B, Applied Biosystems, Foster City, CA). The probes were labeled

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Table I. Analysis of c-Ki-*ras* Mutations at Codons 12 or 13 in Pancreatic Cancer

Case No. (Sample No. in Fig. 1)	Age and Sex	Histological type	% tumor cells in samples	c-Ki-*ras* mutations at codons 12 or 13[a]	Stage	Survival time (months)[b]	Smoking[c]
1 (1)	49 M	moderately	>80	asp	IV	20	−
2 (2)	63 F	moderately	>80	asp	IV	25	+
3 (3)	64 F	adenosquamous[d]	>80	val	IV	3	−
4 (4)	63 M	adenosquamous	>80	(−)[f]	IV	3	+
5 (5)	57 M	moderately	>80	asp	IV	10.3	+
6 (6)	65 M	moderately[d]	>80	asp	IV	4	−
7 (7)	75 F	well	>80	val	IV	10	−
8 (8)	52 F	moderately	>80	asp	IV	8	+
9 (9)	70 M	moderately	>80	val	III	7	−
10 (10)	72 F	moderately	>80	val	IV	4	−
11 (11)	74 F	mucinous	<5	—[g]			
12 (12)	77 F	moderately	<5	—	IV	3	−
(35)			>80	asp			
13 (13)	75 F	moderately	<5	—	IV	4	−
(47)			>80	asp			
14 (14)	70 F	mucinous	50	asp	II	12	+
15 (15)	67 M	well	50	asp	IV	5.5	+
16 (16)	48 M	adenoca.[d]	50	asp	IV	3	+
17 (17)	77 F	well	80	cys	II	30[i]	−
18 (18)	70 M	papillary	30	asp	III	12	−
19 (19)	59 M	moderately	80	arg	IV	6.7	−
20 (20)	55 F	papillary	50	asp	III	19	−
21 (21)	53 F	papillary	80	asp	IV	9.3	−
22 (22)	56 M	well	<5	—	II	10.5	+
(43)			>80	val			
23 (23)	77 M	moderately	<5	—			
24 (24)	56 F	well	50	arg	II	24	−
25 (25)	73 F	moderately	80	asp	III	4.3	−
26 (26)	63 M	moderately	80	val	IV	5	+
27 (27)	75 F	well	<5	—	IV	8	+
(44)			>80	asp, val			
28 (28)	65 M	moderately	80	asp	II	6	−
29 (29)	82 F	moderately	80	asp	IV	2	−
30 (30)	66 F	papillary	80	asp	III	19	−
31 (31)	55 F	moderately	50	asp (asp)[h]	III	4	−
32 (32)	80 M	papillary	80	asp	IV	6	+
33 (34)	55 F	(Vater)[e]	>80	(−)		18	−
34 (36)	79 F	(stomach)[e]	>80	asp, val		3	−
35 (37)	47 F	moderately	>80	asp	III	6[i]	−
36 (38)	67 F	papillary	<5	—			
37 (39)	68 F	moderately	>80	asp	IV	7[i]	−
38 (40)	57 F	adenoca.[d]	60	val	IV	4	−
39 (41)	55 M	moderately	20	asp	III	24	−
40 (42)	49 M	well	>80	val	III	4.5	−
41 (45)	63 M	moderately	>80	(−)	IV	4	+
42 (48)	67 M	(duodenum)[e]	>80	val		8	+
43 (49)	68 F	cystadenoca.	80	(−)	III	0.2	−
44 (50)	55 F	moderately	>80	asp	IV	4.5	+

a) Mutations were at codon 12 of c-Ki-*ras* unless otherwise described.
b) Survival time after operation.
c) History of cigarette smoking: +, smoked; −, never smoked.
d) Samples were obtained from liver metastasis.
e) Pathologically diagnosed as carcinomas of papilla of Vater (case No. 33), of stomach (case No. 34) and of duodenum (case No. 42). These cases were therefore excluded from evaluation. Cases No. 34 and No. 42 were, however, clinically considered to be pancreatic cancers.
f) No mutation detectable. *g*) Not relevant.
h) c-Ki-*ras* 13 mutation was also detected. *i*) Alive as of October 31, 1989.

at the 5′ end using [γ-^{32}P]ATP and T4-polynucleotide kinase.

PCR DNA amplification *in vitro* was performed as described previously.[11,12] Sequences of cross codons 12 and 13 of c-Ki-*ras* utilized as the amplimers (oligonucleotide primers) were as described by Verlaan-de Vries *et al.*[13] The reaction mixture contained 1 μg of chromosomal DNA or tissue pellets from paraffin sections, 0.6 μg of each amplimer, 800 μM dNTPs, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 200 μg/ml BSA, and 2 units of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in a total volume of 100 μl. The mixture was incubated at 95°C for 60 s to denature the DNA and the amplimers were allowed to anneal to the DNA at 48–55°C for 90 s. Primer extension was performed at 72°C for 90 s. Thirty-five to forty cycles of denaturation, hybridization and extension of DNA were repeated by using an automated heat-block (Program Temp Control System PC.500; Astec Inc., Fukuoka).

Dot blot hybridization The method described by Verlaan-de Vries *et al.*[13] was used with slight modifications.[14] A 10 μl aliquot of the amplified DNA was spotted onto nylon filters (Gene Screen Plus; New England Nuclear, Boston, MA). The filters were dried and prehybridized for 3–16 h at 56°C in 3.0 M tetramethylammonium chloride, 50 mM Tris-HCl pH 8.0, 2 mM EDTA, 100 μg/ml of sonicated and denatured salmon sperm DNA, 0.1% SDS and 5×Denhardt's solution, and subsequently hybridized for 1 h at 56°C in the same mixture containing ^{32}P-labeled oligomer probes. The filters were washed twice in 2×SSC, 0.1% SDS for 10 min at room temperature then stringently washed twice in 5×SSC, 0.1% SDS for 10 min at temperatures ranging from 59°C to 71°C. Subsequently, the filters were washed twice in the hybridization buffer without Denhardt's solution and salmon sperm DNA and were incubated for 1 h in the same solution at 59–60°C. The filters were then exposed to Kodak XAR-5 film at −70°C using intensifying screens.

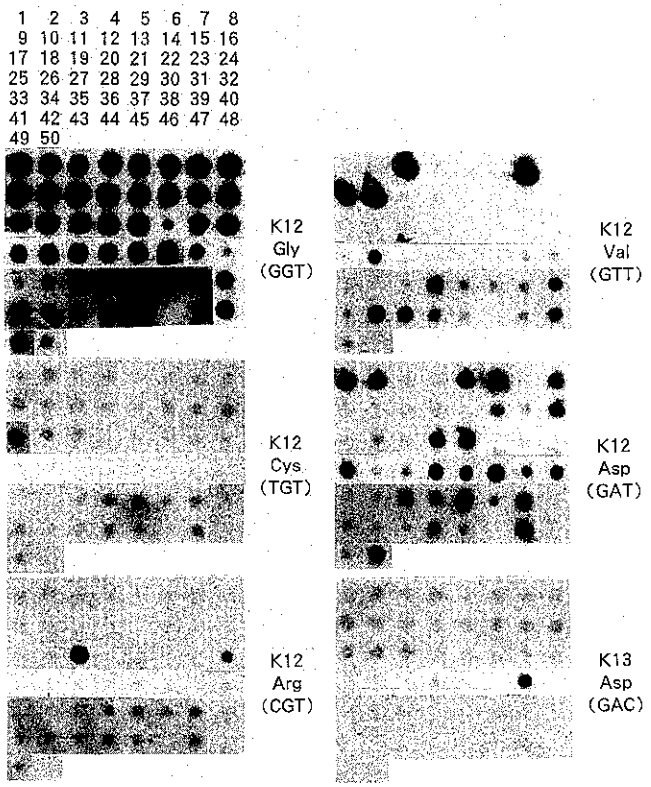

Fig. 1. Detection of point mutations at codons 12 and 13 in c-Ki-*ras* in human pancreatic cancer by dot blot hybridization assays. Autoradiograms of hybridizations in identical dot blots by oligonucleotide probes specific for the normal sequence GGT of codon 12 (Gly), and for mutated sequences TGT (Cys), CGT (Arg), GTT (Val) and GAT (Asp) of codon 12 and GAC (Asp) of codon 13 are shown. Sample numbers follow those in Table I. Two samples (Nos. 33 and 46) are blank.

RESULTS

DNAs obtained from either frozen tissues or paraffin-embedded tissue specimens of pancreatic cancer were amplified by means of PCR using the primers previously described.[14] The presence of point mutations at codons 12 and 13 was examined by dot blot hybridization assays utilizing the synthesized oligonucleotide probes.[14] Forty-one cases of pancreatic cancer were analyzed and the results are shown in Fig. 1 and Table I.

In three cases (Nos. 11, 23 and 36) the samples did not contain sufficient tumor cells to allow detection of mutations by our procedure. In our standardized analysis we were able to detect point mutations with confidence when mutations were present in one allele in more than 10% of the cells (data not shown). These three cases were therefore excluded from evaluation. Three cases (Nos. 4, 41 and 43) with sufficient tumor cells did not harbor mutations at codon 12 or 13 of the c-Ki-*ras* gene. Thirty-five out of the 38 cases with adenocarcinomas of the exocrine pancreas contained mutations at codons 12 or 13 of the c-Ki-*ras* gene. Two cases (Nos. 27 and 31) contained double mutations; glycine to aspartic acid and valine at codon 12 of c-Ki-*ras* in case 27, and glycine to aspartic acid at codons 12 and 13 of c-Ki-*ras* in case 31.

The spectrum of c-Ki-*ras* mutations observed in our analysis is summarized in Table II. Glycine-to-aspartic acid mutation at codon 12 of c-Ki-*ras* was the most

Table II. Comparative Spectra of c-Ki-*ras* Mutations at Codons 12 and 13 in Pancreatic Cancer

		Nagata *et al.* (Japan)[a]	Smit *et al.* (Netherlands)[a]	Grünewald *et al.* (Austria)[a]
Total patients		n=38	n=30	n=63
Patients with mutations		n=35	n=28	n=47
K12				
Cys	TGT	3%[b]	36%	0%
Ser	AGT	0%	0%	0%
Arg	CGT	5%	4%	31%
Val	GTT	24%	28%	31%
Asp	GAT	65%	32%	37%
Ala	GCT	0%	0%	1%
K13				
Asp	GAC	3%	0%	0%
1st letter mutation		8%	40%	31%
2nd letter mutation		92%	60%	69%
Total mutation				
	G→T	27%	64%	31%
	G→A	68%	32%	37%
	G→C	5%	4%	32%

a) Countries where analyses were performed.
b) Number of particular mutations/Number of total mutations×100%.

Table III. The Relationship between Cigarette Smoking and c-Ki-*ras* Mutation in Pancreatic Cancer

		Never	Current
K12 or K13			
Cys	TGT	1[a]	0
Arg	CGT	2	0
Val	GTT	6	3
Asp	GAT	15	9
No mutation		1	2
1st letter mutation		3	0
2nd letter mutation		21	11
Total mutation			
	G→T	7	3
	G→A	15	9
	G→C	2	0

a) Number of patients.

frequent, accounting for 65% of all detected mutations, and the frequency of mutation to valine at the same codon was 24%. Other type of mutations were much less frequent. Data from previous reports by Smit *et al.*[7] and Grünewald *et al.*[8] are also listed in Table II for comparison. In our analysis, neither the presence of mutations at these particular codons of c-Ki-*ras* nor the pattern of mutations seemed to be correlated with the histological type or stage of tumors, or the survival time of patients.

The spectra of c-Ki-*ras* mutations in pancreatic cancer of cigarette smokers and non-smokers were also compared (Table III). No significant difference between two groups was observed.

DISCUSSION

In accordance with recent reports by other investigators,[6-8] we also found a high incidence of point mutations at codon 12 of c-Ki-*ras* in pancreatic cancer. This strongly indicates an essential role of such mutation in human pancreatic cancer.

Several points, however, need to be discussed in comparison with the previous results. Table II summarizes the incidences and the patterns of c-Ki-*ras* mutations in pancreatic cancer reported by Smit *et al.*[7] and Grünewald *et al.*,[8] and also by us. Although all these analyses indicate a high incidence of c-Ki-*ras* mutations at codon 12, the nature of the mutations seems to be quite diverse. In our analysis the amino acid change of glycine to aspartic acid as a consequence of the G-to-A transition of the 2nd nucleotide was observed in about two-thirds of all cases with mutation. A change to valine was found in the remaining one-third. On the other hand, Smit *et al.* found mutations leading to cysteine, valine and aspartic acid essentially with equal incidences, whereas Grüne-

wald *et al.* found mutations leading to arginine, valine and aspartic acid almost equally. Recently, Mariyama *et al.*[15] also reported that 8 out of 12 Japanese pancreatic cancer patients possessed glycine-to-aspartic acid transitions as a consequence of mutations at codon 12 of c-Ki-*ras*, which is closely similar to our findings. The question arises, what brought about the difference in mutation sites in pancreatic cancers among these reports, even though all the results share the common characteristic of dominant mutations at codon 12 of c-Ki-*ras* ? Genetic factors may affect the mutation site of c-Ki-*ras* or the susceptibility to activated c-Ki-*ras* gene products post-transcriptionally, either of which eventually results in clonally expanded pancreatic cancer cells with distinct activated c-*ras* genes. In the former case, the frequencies and types of mutations are solely determined at the DNA level, in which case an amino acid change itself is more crucial than the nature of the altered amino acid. Alternatively, in the latter case, particular types of amino acid changes at these codons could result in greater potential transforming activities than other types of amino acid changes. Since distinct transitions and transversions of the first and second nucleotides at codon 12 of c-Ki-*ras* result in different amino acid changes, either explanation is possible. The recent report by Newcomb *et al.* suggested that the genetic background of murine hosts influenced the preference of c-*ras* mutations in radiation-induced or chemically induced tumors.[16]

Non-genetic influences also have to be considered. In this regard, smoking is currently the only generally accepted high risk factor for pancreatic cancer.[17] Smit *et al.* stressed in their analysis that the modes of c-Ki-*ras* mutations in pancreatic cancer are somewhat reminiscent of those in lung cancers; in both of these cancers G-to-T transversion at codon 12 is dominant, but not in colon cancer.[7] In our analysis, however, pancreatic cancers which developed in cigarette smokers and non-smokers displayed no significant difference of mutational pattern in c-Ki-*ras*, as shown in Table III. It seems to be rather unlikely that cigarette smoking, which could be involved in pancreatic oncogenesis, directly influences the preference for mutations at codon 12. For the interpretation of preferential mutation patterns, various genetic and non-genetic factors may have to be taken into account.

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