RAPID COMMUNICATION Jpn. J. Cancer Res. 80, 622–626, July 1989

Frequency and Types of Point Mutation at the 12th Codon of the c-Ki-*ras* Gene Found in Pancreatic Cancers from Japanese Patients

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Point mutations at the 12th codon of c-Ki-*ras* in pancreatic cancer from Japanese patients were examined using the polymerase chain reaction, followed by cloning of the amplified gene fragments in pTZ phagemid and nucleotide sequence determination. The frequency of the point mutations found in the tumors was quite high (75%). The mutation most frequently detected was a G→A transition at the second position of codon 12 (GGT→GAT), but other types of mutations such as GGT→GTT and GGT →CGT were also found. In one case, silent mutation of GGT to GGC was detected in addition to the frequent mutation of GGT to GAT. These observations suggest that the 12th codon of pancreatic c-Ki-*ras* is highly mutatable.

Key words: c-Ki-*ras* — Polymerase chain reaction — Pancreas — Adenocarcinoma — Silent mutation

Point mutations of *ras* family genes (c-Ha-*ras*, c-Ki-*ras* and N-*ras*) at either the 12th, 13th or 61st codon are the oncogene activations most frequently found in a variety of human cancers, and are believed to be relevant to induction and/or progression of human cancers.[1–3] We previously showed that human pancreatic cancer contains activated c-Ki-*ras* having a point mutation at the 12th codon (GGT→CGT; Gly→Arg).[4, 5] In addition it was shown that activated c-Ki-*ras* and c-*myc* were amplified in this particular pancreatic cancer.[5] Subsequently point mutation of c-Ki-*ras* at the 12th codon has been found to be extraordinarily frequent in human cancers of the exocrine pancreas; in 21 out of 22 cases, such a point-mutated c-Ki-*ras* was detected.[6] This is very unusual, since the frequency of point mutations of *ras* family genes in human cancers is in general low; only between 5 and 15% of tumors examined, except colon cancer, acute myeloid leukemia and myelodysplastic syndrome.[7–13]

One of our aims in analyzing c-Ki-*ras* activation in human pancreatic cancer was to confirm Perucho and his colleagues' observation by examining whether the same extent of point mutation of c-Ki-*ras* is observed in pancreatic cancer in Japanese patients. Secondly, in contrast to their report,[6] we attempted to determine precisely the type of mutation at the 12th position of c-Ki-*ras* using the polymerase chain reaction (PCR), followed by cloning into pTZ phagemid and subsequent nucleotide sequence determination.

Paraffin-embedded tissue blocks were prepared from surgical specimens of pancreatic cancers taken from patients who had undergone operations at the National Cancer Center Hospital. Their age and sex and pathological data of the pancreatic cancers are given in Table I. DNA was extracted from the paraffin sections by the procedure described by Shibata *et al.*[14] Namely, two 5 μm sections were cut out from the paraffin-embedded block. One of them was stained with hematoxylin-eosin in order to locate precisely the region of cancerous tissue by microscopic examination. By comparing hematoxylin-eosin-stained sections, the cancerous portion was cut from another 5 μm section. The surface area of the tissue was approximately 0.5 cm^2. The tissue was deparaffinized by treatment with xylene and precipitated by centrifugation. The tissue was then treated with 95% ethanol and again precipitated by centrifugation. The tissue pellet thus obtained was dried *in vacuo*, and subjected to PCR.

Amplification of DNA corresponding to the 12th codon of c-Ki-*ras* was performed by the PCR technique developed by Saiki *et al.*[15] The two oligonucleotides used as primers for PCR are as follows: Fragment A, TGGATCCGTGACATGTTCTAATATAGT and Fragment B, TGAATTCAGAGAAACCTTTATCTGTAT. These two primers, A and B, contained restriction endonuclease cutting sites for *Bam*HI and *Eco*RI, respectively. Therefore, after amplification, the amplified DNA was treated with *Bam*HI and *Eco*RI, and ligated to a pTZ vector. Then, the amplified DNAs were cloned and

Table I. Age and Sex of 12 Patients with Pancreatic Cancer and Site of Origin and Pathologic Type of Their Cancer

Patient No.	Age (yr)	Sex	Lesion	Pathological type
1	66	M	Head	Tubular adenoca., moderately diff.
2	75	F	Head-Tail	Tubular adenoca., moderately diff.
3	63	F	Body-Tail	Tubular adenoca., moderately diff.
4	59	M	Body-Tail	Papillotubular adenoca., well diff.
			Lymph node	Tubular adenoca., well diff.
5	58	F	Head	Tubular adenoca., moderately diff.
6	71	F	Head	Tubular adenoca., poorly diff.
7	77	M	Head	Tubular adenoca., well diff.
8	58	F	Seeding	Adenoca.
9	75	F	Head	Tubular adenoca., moderately diff.
			Lymph node	Tubular adenoca., poorly diff.
10	72	F	Head	Papillotubular adenoca., well diff.
11	61	M	Head	Papillotubular adenoca., well diff.
12	76	M	Head	Papillotubular adenoca., well diff.

Table II. Type and Frequency of Point Mutation at the 12th Codon of c-Ki-*ras* in Pancreatic Cancer

Patient No.	$\frac{\text{Cancerous tissue}}{\text{Total tissue area}} \times 100\%$	Mutated clones/Total clones examined (%)	Type of mutation	Remarks
1	40	2/11 (18)	GGT→GTT (Gly→Val)	
2	50	2/12 (17)	GGT→CGT (Gly→Arg)	
3	70	1/10 (10)	GGT→GTT (Gly→Val)	
4a	60	0/14 (0)		
4b	20	1/5 (20)	GGT→GAT (Gly→Asp)	lymph node metastasis
5	50	4/19 (21)	GGT→GAT (Gly→Asp)	
6	30	0/17 (0)		
7	20	0/10 (0)		
8	ND[a]	6/11 (55)	GGT→GAT (Gly→Asp)	
9a	80	18/28 (57)	GGT→GAT (Gly→Asp)	
〃	〃	1/28 (4)	GGT→CGT (Gly→Arg)	
〃	〃	1/28 (4)	GGT→GGC (Gly→Gly)	
9b	80	4/7 (57)	GGT→GAT (Gly→Asp)	lymph node metastasis
10	40	13/17 (76)	GGT→GAT (Gly→Asp)	
11	60	16/16 (100)	GGT→GAT (Gly→Asp)	
12	50	8/13 (62)	GGT→GAT (Gly→Asp)	

In cases No. 4 and 9, the analysis was made by using two different specimens (a and b) from the same tumor. In case No. 8, fresh tissue was used for the analysis. Approximate ratio of cancerous tissue to total area of the specimen, which contains normal fibroblasts, lymphocytes, plasma cells, pericytes of capillaries and endothelial cells of capillaries, was determined by microscopic pathological examination of the hematoxylin-eosin-stained section made from one of the two 5 μm sections.

a) ND, not determined.

sequenced by the dideoxy chain termination method. By adopting this procedure, point mutations of the c-Ki-*ras* gene at the 12th codon can be clearly identified. In addition, contrary to the oligonucleotide hybridization method[16] or the RNAase A mismatch cleavage method,[6] mutations present in a small fraction of the gene can be clearly detected by this procedure. In order to get data which are more meaningful, in most cases we have sequenced many clones derived from each specimen, as shown in Table II. Figures 1 and 2 show typical examples of sequencing gels in which the point mutations at the 12th codon are clearly seen. By analyzing 12 cases of pancreatic cancer as summarized in Table II, the following results were obtained.

Firstly, point mutations of c-Ki-*ras* at the 12th codon were detected with high frequency in pancreatic cancer of Japanese patients, in agreement with the report by Almoguere *et al.*[6] Out of 12 cases, 9 cases were found to have point mutations (75%). The point mutation most often found is a G→A transition at the second position of the 12th codon, resulting in alteration of glycine to aspartic acid (5 cases). But, other types of mutation such as GGT→GTT (Gly→Val) and GGT→CGT (Gly→Arg) were also found. It is interesting to note that the ratio of the clones having the point mutation to those without the mutation varied significantly with each pancreatic cancer (Table II). For example, in case No. 11, the point mutation (GGT→GAT) was found with 100% frequency, whereas in case No. 3, the point mutation (GGT→CGT) was found with about 10% frequency. This variation does not seem to be due to the extent of contamination of normal tissues in the cancer cells analyzed, since in both cases the contamination was found to be approximately the same (Table II). No correlation was found between the type and extent of the mutation and the pathological status of the cancers. The result obtained with case No. 11 suggested that one allele of c-Ki-*ras* is deleted, or that point-mutated c-Ki-*ras* is extensively amplified. In case No. 4, the point mutation was only found in cancer cells in lymph nodes metastasized from the original pancreatic cancer. This result together with the observation of the low frequency of point mutations in some pancreatic cancers suggests that the point mutation of c-Ki-*ras* at the 12th codon in pancreatic cancer is not a causative event of pancreatic cancer, but a later event.

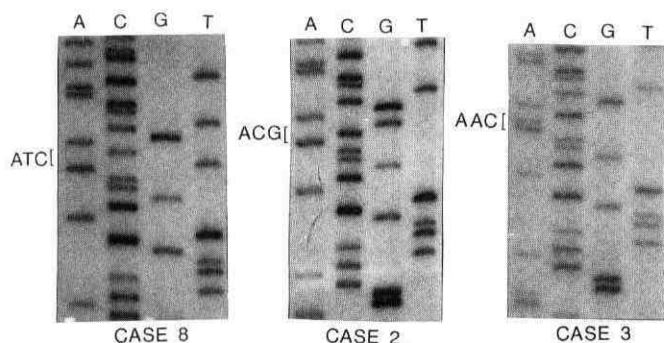

Fig. 1. Autoradiograms of sequencing gels showing the point mutation in the region of the 12th codon of c-Ha-*ras* in human pancreatic cancer. The sequence of the sense strand was determined. Case No. 8, GGT→GAT mutation; case No. 2; GGT→GTT mutation; case No. 3, GGT→GTT mutation. The reaction mixture (100 μl) for PCR contained 1 μM each of the primer, 200 μM each of dNTPs, 10 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 50 mM KCl, 0.1% gelatin and 1 μg of DNA or tissue pellet. The mixture was incubated for 5 min at 98°C and then subjected to 40 cycles of amplification with 2.5 units of Taq polymerase (Perkin-Elmer-Cetus). Each cycle consisted of an incubation of 1 min at 90°C, 2 min at 55°C and 2.5 min at 70°C. The reaction products were purified by 8% polyacrylamide gel electrophoresis. The purified amplified DNA fragments were digested with *Bam*HI and *Eco*RI, and ligated into a pTZ vector as described previously.[20] The cloned DNAs were sequenced with Sequenase™ using the dideoxy chain termination method.[21]

Supposing that the activation of c-Ki-*ras* by point mutation at the 12th codon is important for malignancy or progression of human pancreatic cancer, a question arises as to the situation in large portions of the cancer cells in which the point mutation does not occur in c-Ki-*ras* at the 12th codon. It is possible that pancreatic

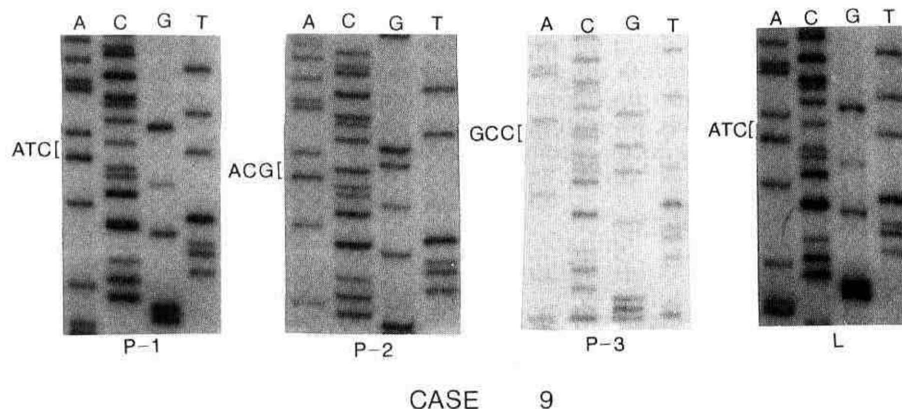

Fig. 2. Sequencing autoradiograms of the complementary strand of the region of the 12th codon of c-Ki-*ras* in case No. 9, showing three point mutations at codon 12. P-1, P-2 and P-3, primary carcinoma; L; lymph node metastasis.

cancer in which only a small fraction of c-Ki-*ras* is mutated at the 12th codon may have additional mutations in c-Ki-*ras* or other *ras* family genes, thus making the cells malignant. In fact, Hirai *et al.*[17] previously reported that the human pancreatic cancer cell line T3M-4 contained c-Ki-*ras* having a mutation at the 61st codon.

It is believed that cancers are in general mixtures of subclones derived from the original clone, although no experimental evidence is available in the case of pancreatic cancer. Therefore, in order to clarify the relationship between point mutational activation of c-Ki-*ras* and induction and/or progression of pancreatic cancer, further studies are needed to analyze the status of c-Ki-*ras* in many cancerous tissues separately localized in pancreas cancer.

In case No. 9, additional mutations (GGT→CGT and GGT→GGC) were found, although the frequencies of these mutations are low. Surprisingly, one of those mutations was silent, i.e. it does not cause amino acid alteration. Since such a silent mutation should not give any growth advantage to cells containing it, this observation suggested that the 12th codon of c-Ki-*ras* in the pancreas is a "hot spot" where mutation occurs with high frequency. It should be noted that the silent mutation GGT→GGC has also been detected in portions of normal pancreatic tissues of case No. 5 (1 out of 2 clones) and case No. 6 (1 out of 4 clones)(unpublished results). At present the possibility that modification of DNA occurs during fixation of the tissue by formaldehyde, and that such modification causes the silent mutation as an artifact is not completely excluded.

The results reported here are not consistent with the previous report by Almoguera *et al*,[6] who concluded that the mutated c-Ki-*ras* gene is present in most of the neoplastic cells and that c-Ki-*ras* mutational activation is an early event in carcinogenesis of the human pancreas. The discrepancy with our results may be due to the procedure they employed. They used PCR followed by the RNAase A mismatch cleavage method to detect the mutation, thus making it difficult to get a quantitative estimation of the amount of mutated c-Ki-*ras*, because they reported that incomplete cleavage of single base mismatches present in DNA-RNA hybrids was possible.

Recently Smit *et al.*[18] have reported the finding of c-Ki-*ras* in pancreatic adenocarcinoma. They used oligonucleotide hybridization after PCR, and found that c-Ki-*ras* mutations in the 12th codon are mainly G→T transversions at the first or second base (GGT→TGT or GGT→GTT), along with a G→A transition at the second base. Our results are rather similar to theirs, but differ in that we observed the G→A transition more frequently. Although they found a weak mutation-specific signal in addition to the strong mutation-specific signal in a single preparation, they are unable to draw firm conclusions as to the presence of multiple mutations because of the limitations of the oligonucleotide hybridization technique. Our results, obtained by cloning and sequencing, clearly prove that multiple mutations are present.

Neri *et al.*[19] reported that in the case of acute lymphoblastic leukemia, N-*ras* mutations are found in only a fraction of malignant cells, suggesting that selective loss or acquisition of the mutated alleles happens to occur during tumor development. They also reported that two distinct N-*ras* mutations were present in a single specimen of the tumor. These results are compatible with the results on pancreatic cancer as reported in this communication.

This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy for Cancer Control and for Cancer Research. M.M. is a research resident supported by the Foundation for Promotion of Cancer Research (Tokyo).

(Received April 15, 1989/Accepted May 22, 1989)

REFERENCES

1) Nishimura, S. and Sekiya, T. Human cancer and cellular oncogenes. *Biochem. J.*, **243**, 313–327 (1987).
2) Barbacid, M. *ras* Genes. *Ann. Rev. Biochem.*, **56**, 779–827 (1987).
3) Bos, J. L. The *ras* gene family and human carcinogenesis. *Mutat. Res.*, **195**, 225–271 (1988).
4) Prassolov, V. S., Sakamoto, H., Nishimura, S., Terada, M. and Sugimura, T. Activation of c-Ki-*ras* gene in human pancreatic cancer. *Jpn. J. Cancer Res.*, **76**, 792–795 (1985).
5) Yamada, H., Sakamoto, H., Taira, M., Nishimura, S., Shimosato, Y., Terada, M. and Sugimura, T. Amplifications of both c-Ki-*ras* with a point mutation and c-*myc* in a primary pancreatic cancer and its metastic tumors in lymph nodes. *Jpn. J. Cancer Res.*, **77**, 370–375 (1986).
6) Almoguera, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N. and Perucho, M. Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes. *Cell*, **53**, 549–554 (1988).
7) Bos, J. L., Fearon, E. R., Hamilton, S. R., Verlaan-de Vries, M., van Boom, J. H., van der Eb, A. J. and Vogelstein, B. Prevalence of *ras* gene mutations in human colorectal cancers. *Nature*, **327**, 293–297 (1987).
8) Forrester, K., Almoguera, C., Han, K., Grizzle, W. E. and

Perucho, M. Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. *Nature,* **327,** 298–303 (1987).

9) Bos, J. L., Toksoz, D., Marshall, C. J., Verlaan-de Vries, M., Veeneman, G. H., van der Eb, A. J., van Boon, J. H., Janssen, J. W. G. and Steenvoorden, A. C. M. Amino-acid substitutions at codon 13 of the N-*ras* oncogene in human acute myeloid leukemia. *Nature,* **315,** 726–730 (1985).

10) Bos, J. L., Verlaan-de Vries, M., van der Eb, A. J., Janssen, J. W. G., Delwel, R., Löwenberg, B. and Colly, L. P. Mutations in N-*ras* predominate in acute myeloid leukemia. *Blood,* **69,** 1237–1241 (1987).

11) Farr, C. J., Saiki, R.K., Erlich, H. A., McCormick, F. and Marshall, C. J. Analysis of *RAS* gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes. *Proc. Natl. Acad. Sci. USA,* **85,** 1629–1633 (1988).

12) Hirai, H., Kobayashi, Y., Mano, H., Hagiwara, K., Maru, Y., Omine, M., Mizoguchi, H., Nishida, J. and Takaku, F. A point mutation at codon 13 of the N-*ras* oncogene in myelodysplastic syndrome. *Nature,* **327,** 430–432 (1987).

13) Liu, E., Hjelle, B., Morgan, R., Hecht, F. and Bishop, J. M. Mutation of the Kirsten-*ras* proto-oncogene in human preleukaemia. *Nature,* **330,** 186–188 (1987).

14) Shibata, D. K., Arnheim, N. and Martin, W. J. Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. *J. Exp. Med.,* **167,** 225–230 (1988).

15) Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science,* **239,** 487–491 (1988).

16) Bos, J. L., Verlaan-de Vries, M., Jansen, A. M., Veeneman, G. H., van Boom, J. H. and van der Eb, A. J. Three different mutations in codon 61 of the human N-*ras* gene detected by synthetic oligonucleotide hybridization. *Nucleic Acids Res.,* **12,** 9155-9163 (1984).

17) Hirai, H., Okabe, T., Anraku, Y., Fujisawa, M., Urabe, A. and Takaku, F. Activation of the c-K-*ras* oncogene in a human pancreas carcinoma. *Biochem. Biophys. Res. Commun.,* **127,** 168–174 (1985).

18) Smit, V. T. H. B. M., Boot, A. J. M., Smits, A. M. M., Fleuren, G. J., Cornelisse, C. J. and Bos, J. L. KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas. *Nucleic Acids Res.,* **16,** 7773–7782 (1988).

19) Neri, A., Knowles, D. M., Greco, A., McCormic, F. and Dalla-Favera, R. Analysis of RAS oncogene mutations in human lymphoid malignancies. *Proc. Natl. Acad. Sci. USA,* **85,** 9268–9272 (1988).

20) Mead, D. A., Skorupa, E. S. and Kemper, B. Single stranded DNA SP6 promoter plasmids for engineering mutant RNAs and proteins: synthesis of a 'stretched' preproparathyroid hormone. *Nucleic Acids Res.,* **13,** 1103–1118 (1985).

21) Sanger, F., Nicklen, S. and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA,* **74,** 5463–5467 (1977).