Jpn. J. Cancer Res. 85, 1005–1014, October 1994

K-*ras* and p53 Alterations in Genomic DNA and Transcripts of Human Pancreatic Adenocarcinoma Cell Lines

Hirofumi Suwa,[1,2] Tsunehiro Yoshimura,[1,2] Nozomi Yamaguchi,[3] Kazunori Kanehira,[1] Tadao Manabe,[2] Masayuki Imamura,[2] Hiroshi Hiai[1] and Manabu Fukumoto[1,4]

[1]Department of Pathology and [2]Department of Surgery, Faculty of Medicine, Kyoto University, Yoshida-Konoecho, Sakyo-ku, Kyoto 606, and [3]Department of Cell Biology, Research Institute for Neurological Diseases and Geriatrics, Kyoto Prefectural University of Medicine, Kawaramachi, Hirokoji, Kamigyo-ku, Kyoto 602

We analyzed 15 human pancreatic adenocarcinoma cell lines for alterations of the K-*ras* and the p53 genes and their transcripts. In 11 cell lines (73.3%), point mutations of the K-*ras* gene were found at codon 12 in exon 1. In 9 cell lines one allele was mutated and the other was wild type, and both the alleles were expressed into mRNA. In one cell line both alleles of codon 12 were mutated to TGT and GTT, respectively, but only TGT was transcribed into mRNA. Alterations in mRNA of the p53 gene were detected in 10 cell lines (66.7%). Analysis of the genomic sequence of the p53 gene revealed that the alterations consisted of 6 cases of base pair substitutions and 1 case of 1-bp deletion in evolutionarily conserved exons 5 to 8, 2 cases of splicing mutations in exon 4, and 1 case of novel deletion from exons 2 to 9. In 14 cell lines (93.3%), alterations were identified in the K-*ras* or p53 gene. Of these, 4 cell lines harbored K-*ras* mutations without p53 alteration, whereas 3 cell lines exhibited p53 alterations without K-*ras* mutation. Thus, it is suggested that activation of the K-*ras* gene and inactivation of the p53 gene are strongly and cooperatively associated with pancreatic carcinogenesis.

Key words: K-*ras* — p53 — Genomic DNA — mRNA — Mutation

Pancreatic adenocarcinoma is the fifth leading cause of death from malignant diseases in Japan, as well as in Western countries.[1)] It carries an extremely poor prognosis; fewer than 20% of patients survive the first year, and only 3% are alive five years after the diagnosis.[2)] The high mortality rate of pancreatic carcinoma is attributed to several factors, such as its biologically aggressive nature, difficulties in early detection, inadequacy of surgical resection, and refractoriness to other treatments including chemotherapy and radiotherapy.[1,3)]

In recent years it has become apparent that malignant transformation is a result of accumulation of genetic abnormalities, including activation of oncogenes and inactivation of tumor suppressor genes.[4)] Finding molecular genetic changes which are characteristic of pancreatic adenocarcinoma will contribute to the elucidation of the pathogenesis and to the possibility of early detection of pancreatic carcinoma. Activation of the K-*ras* oncogene by point mutations at codon 12 is detected very frequently in human pancreatic carcinomas.[5–8)] Further, K-*ras* activation is an early event in N-nitroso-bis(2-oxopropyl)amine-induced pancreatic carcinogenesis in the hamster.[9,10)] These observations suggest that K-*ras* activation is a critical step in pancreatic carcinogenesis.[11)] Inactivation of the p53 tumor suppressor gene is frequently involved in carcinogenesis of various organs including colon, lung, breast, liver, ovary and brain.[12,13)] Recently mutations in exons of the p53 gene accompanied with overexpression of the p53 protein detected by immunocytochemistry and concomitantly frequent K-*ras* point mutations, have been reported both in tumor tissues and in cell lines of human pancreatic carcinoma.[3,14–16)]

The aim of the present study was to investigate the involvement of the K-*ras* and p53 genes in the pathogenesis of pancreatic adenocarcinoma at the transcriptional level into mRNA as well as in genomic DNA. Alteration of expressed mRNA rather than genomic DNA is more informative for evaluation of the biological relevance of these genes. It is difficult to obtain fresh surgical tissue specimens from pancreatic adenocarcinoma because of the frequency of unresectable cases. Furthermore, the characteristic that pancreatic carcinoma consists of a great majority of non-malignant cells, because of severe fibrosis, results in underestimation of molecular genetic changes. Consequently we used cell lines derived from human pancreatic carcinoma to analyze molecular genetic changes precisely. In addition, p53 protein was stained immunocytochemically to look for any relation between staining intensity and p53 gene mutations.

[4] To whom correspondence and reprint requests should be addressed.

[5] Abbreviations: PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; SSCP, single strand conformation polymorphism.

MATERIALS AND METHODS

Cell lines Each cell line was established from either a primary tumor or a metastatic lesion of human pancreatic carcinomas. Cell lines Capan-2, HS-700T, BXPC-3, MIAPaCa-2, Panc-1 were from the American Type Culture Collection (Rockville, Maryland). Other cell lines were established by one of us.[17] The clinicopathological data of the patients from whom cell lines were derived, are presented in Table I.

Oligonucleotide primers and probes for PCR For amplification of *ras* gene family members, PCR[5] primer and probe kits were purchased from Takara Shuzo (Kyoto). For amplification of the entire coding region of the p53 cDNA, a pair of primers was synthesized according to Nigro *et al.*[12] The sequences of synthesized PCR primers specific for each exon from 4 to 9 of the p53 gene are listed in Table II.

PCR reaction DNA and RNA were extracted from each cell line by the methods previously described.[18, 19] Complementary DNA was prepared by random priming from total RNA using a First-Strand cDNA Synthesis kit (Pharmacia-LKB, Sweden). For amplification of *ras* gene family members, PCR was performed according to the manufacturer's protocol. For p53 mRNA analysis, partial fragments were further amplified from the entire coding region of cDNA using exon-specific primers. For analysis of the p53 gene, genomic DNA instead of cDNA was used as the template for PCR. For determination of the copy number of the K-*ras* gene, primer sets for the K-*ras* gene and the S26 gene[20] were used. The PCR mixture contained 25 pmol of each primer, 1.25 mmol of dNTP, and 0.5 U of *Taq* DNA polymerase with reaction buffer (Wako Chemicals, Tokyo) to a final volume of 10 μl. As a template, cDNA from 50 ng of total RNA or 100 ng of genomic DNA was added to the mixture. The conditions of PCR were as follows; denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at optimal temperature for each pair of primers between 55 and 66°C for 2 min, extension at 72°C for 1 min in a thermal cycler (Perkin-Elmer Cetus, Connecticut). For detection of gross deletions in the p53 mRNA, the RT-PCR products of p53 were electrophoresed in 1% agarose gel and stained with ethidium bromide. For measurement of copy number, 37 kBq of ^{32}P-dCTP was added to the PCR mixture. PCR products after 20 cycles were separated by 12% polyacrylamide gel and incorporated radioactivity of the bands was measured by a BAS 2000 image analyzer (Fujix, Tokyo).

Slot blot hybridization of the K-*ras* gene Slot blot hybridization to detect point mutations of the K-*ras* gene and cDNA was performed as previously described.[18]

Southern hybridization of the p53 gene Five micrograms of genomic DNA digested with *Pst*I was subjected to 1% agarose gel electrophoresis followed by transfer onto a nylon membrane. The membrane was hybridized to a p53 full-length cDNA probe.[21]

Direct sequencing analysis of the p53 and K-*ras* gene Either of the PCR primers was labeled with ^{32}P-ATP by polynucleotide kinase, and sequencing was carried out using a Circumvent DNA Sequencing kit (New England Biolab, Maryland) in a thermal cycler according to the manufacturer's protocol. Samples were denatured at 90°C for 3 min and applied to 8% polyacrylamide gel containing 8.3 mol/liter urea. Electrophoresis was performed at a constant power of 20 W for 2–6 h at room temperature. The gel was dried and an autoradiogram was made for analysis.

Table I. Cell Lines Investigated

Cell line	Age	Sex	Origin	Histology	Source of culture	Xenotransplantation
HPC-Y1	58	M	Ductal cell carcinoma	W[a]	Primary tumor	–
HPC-Y3	66	M	Ductal cell carcinoma	W	Primary tumor	+
HPC-Y5	57	M	Ductal cell carcinoma	W	Lymph node metastasis	+
HPC-Y11	60	M	Ductal cell carcinoma	W	Primary tumor	+
HPC-Y19	59	M	Ductal cell carcinoma	M[b]	Primary tumor	+
HPC-Y21	61	M	Ductal cell carcinoma	W	Primary tumor	+
HPC-Y23	55	M	Ductal cell carcinoma	W	Primary tumor	+
HPC-Y25	53	M	Ductal cell carcinoma	W	Primary tumor	+
Capan-2	56	M	Adenocarcinoma		Liver metastasis	+
HS-700T	61	M	Adenocarcinoma		Liver metastasis	ND[d]
HPC-YO	62	M	Acinar cell carcinoma		Primary tumor	+
HPC-YT	64	M	Ductal cell carcinoma	W	Primary tumor	+
BXPC-3	61	F	Ductal cell caricnoma	P[c]	Primary tumor	+
MIAPaCa-2	65	M	Undifferentiated carcinoma		Primary tumor	ND
Panc-1	56	M	Anaplastic carcinoma		Primary tumor	+

a) Well differentiated. *b*) Moderately differentiated. *c*) Poorly differentiated. *d*) Not done.

Table II. PCR Primers

Gene		Primer	PCR product (bp)
p53 genomic DNA	Exon 4-1	U CGTTCTGGTAAGGACAAGGG	199
		D TCTGGGAGCTTCATCTGGA	
	Exon 4-2	U CCCGGACGATATTGAACAAT	275
		D GAAGTCTCATGGAAGCCAGC	
	Exon 5	U TACTCCCCTGCCCTCAACAAG	196
		D CCAGCTGGCACCATCGCTA	
	Exon 6	U GAGCACTGCTCAGATAGCGAT	216
		D GTGCCCTATGAGCCGCCTGAG	
	Exon 7	U GTTGGCTCTGACTGTACCACC	111
		D ATCACACTGGAAGACTCCAGT	
	Exon 8	U CTGCCTCTTGCTTCTCTTTT	204
		D TCTCCTCCACCGCTTCTTGT	
p53 cDNA	Exon 4-A	U GAGCCGCAGTCAGATCCTA	294
		D CTGGGAAGGGACAGAAGATG	
	Exon 4-B	U AGAATGCCAGAGGCTGCTC	227
		D GTCTTGGCCAGTTGGCAA	
	Exon 5	U CTGTGACTTGCACGTACTCC	232
		D ACTCGGATAAGATGCTGAGG	
	Exon 6	U CATGAGCGCTGCTCAGATAG	160
		D TGGTGGTACAGTCAGAGCCA	
	Exon 7	U TCGACATAGTGTGGTGGTGC	230
		D GATTCTCTTCCTCTGTGCGC	
	Exon 8, 9	U TCATCACACTGGAAGACTC	271
		D TCTCGGAACATCTCGAAGCG	
S26		U GGTCCGTGCCTCCAAGATGA	99
		D CCAAGGACAAGGCCATTAAG	

Immunocytochemical analysis of the p53 protein Cells were grown on glass slides and were fixed with 10(v/v)% formalin for 20 min at room temperature. After washing with phosphate-buffered saline, the glass slides were incubated overnight at 4°C with a mouse monoclonal antibody, DO-7,[22] which recognizes amino acid residues 1–45 of the p53 protein. Antibody localization was determined using a Vectastain ABC Elite kit (Vector Lab., California) after application of biotinylated horse anti-mouse antibody.

RESULTS

Mutations of the K-*ras* gene Mutations of the H-*ras* and the N-*ras* genes in genomic DNA were not detected. On the other hand, K-*ras* mutation was found in 11 (73.3%) of 15 cell lines by SSCP analysis (data not shown). In order to determine patterns of mutations and allele-specific transcription of the K-*ras* gene, slot blot hybridization with mutation-specific oligonucleotide probes was performed (Fig. 1). All of the mutations found were located at codon 12 of exon 1, and no mutation was observed at codons 13 or 61. Three kinds of mutational patterns were found in codon 12: G-T transversion at the second nucleotide in 5 cell lines (41.7%) and at the first nucleotide in 2 cell lines (16.7%), resulting in amino acid

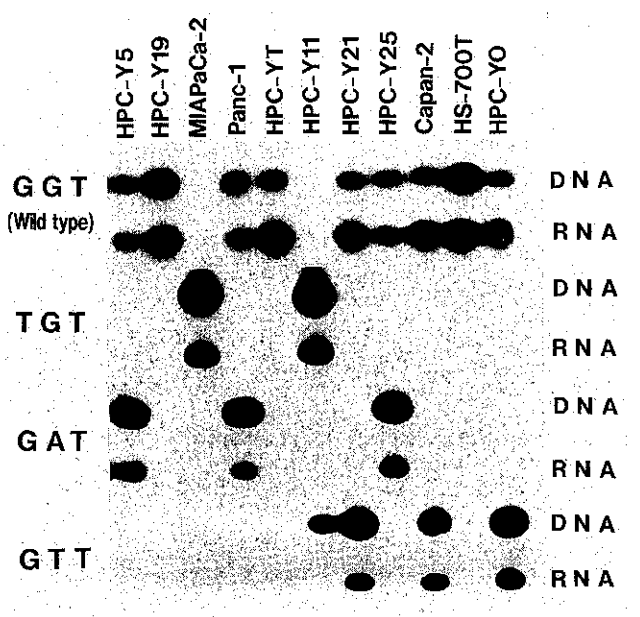

Fig. 1. Slot blot hybridization to mutation-specific probes at codon 12 of the K-*ras* gene. Upper rows are amplified fragments from genomic DNA and lower rows are those from cDNA. In HPC-Y11 cells both the alleles were mutated to TGT and GTT, but only TGT was expressed. MIAPaCa-2 cells contained and expressed only TGT.

changes from glycine to valine and to cysteine, respectively. G-A transition was found at the second nucleotide in 5 cell lines (41.7%), changing glycine to aspartic acid. The cell lines, in which one allele was mutated and the other was wild type, expressed both the alleles into mRNA. The expression levels of the wild-type sequence were about three times higher than those of the mutated sequences in individual cell lines. Interestingly, in HPC-Y11 cells both alleles of codon 12 were mutated, to TGT and GTT, respectively. However, only TGT was expressed into mRNA. MIAPaCa-2 cells contained only mutated codon 12 TGT, which was transcribed into mRNA. The K-*ras* gene in MIAPaCa-2 cells determined by PCR was a single copy per haploid of genomic DNA (Fig. 2). The mutational patterns at codon 12 observed by direct sequencing were in agreement with those observed by slot blot hybridization (data not shown).

Gross deletion of mRNA and genomic DNA in the p53 gene Fig. 3 shows profiles of PCR products after reverse transcription (RT-PCR) from exons 2 through 11 of the p53 mRNA. Three cell lines, HPC-Y1, HPC-Y5, Capan-2 had smaller-sized transcripts compared with the expected size of 1.3 kb. The sequence analysis of RT-PCR products revealed that HPC-Y1 cells had a gross deletion in mRNA from the 5′ end of exon 2 through the 3′ end of exon 9 (Fig. 4A). In HPC-Y5 cells, the whole of exon 4 was deleted in mRNA (Fig. 4B). Capan-2 cells showed a complex mutation consisting of a 200-bp deletion of the 3′ side of exon 4 and a frame shift (Fig. 4C).

In order to clarify whether the deletions of the p53 mRNA in these cell lines were derived from splicing mutations or deletions of corresponding sequences in genomic DNA, Southern blot analysis with the p53 cDNA probe was performed (Fig. 5). In the HPC-Y1 cell line, band B corresponding to exons 10–11 was observed, whereas bands C (exons 2–4) and A (exons 5–9) were not seen. These results indicate that HPC-Y1 cells had a gross deletion from exon 2 to exon 9 of the genomic p53 gene. HPC-Y5 cells had a smaller-sized band C′, which corresponded to partial deletion in exons 2–4. Direct sequencing of the genomic p53 gene revealed a 76-bp deletion in the intron 3-exon 4 junction (Fig. 4B). Thus, splicing out of exon 4 in HPC-Y5 cells was due to the 76-bp deletion including the splicing acceptor site of intron 3. Direct sequencing of genomic DNA revealed that Capan-2 cells did not harbor deletion, but rather revealed G-T base pair substitution at the third nucleotide of codon 125 (Fig. 4C). This point mutation initiated the use of a cryptic splicing donor site within exon 4, resulting in 200-bp deletion of the 3′ side of exon 4 in the mRNA. In addition, a frame shift, which was caused by the abnormal splicing. introduced a termination codon next to 65 bp from the 5′ end of exon 5. A schematic illustration of these splicing mutations is shown in Fig. 6.

Mutations of the p53 gene within exons (Table III) For the purpose of investigating other gene alterations in the p53 gene and mRNA, apart from the gross deletions mentioned above, we performed direct sequencing with PCR products of both genomic DNA and cDNA in 12 other cell lines. We focused on the sequence analysis from exons 4 to 9, because point mutations of the p53 gene preferentially occur between exons 5 and 8, which correspond to an evolutionarily conserved region.[13] Missense mutations involving 1-base substitution were found in 6 cell lines. Patterns of transition found were G-A in 2 cell lines, A-G in 2 cell lines and C-T in 1 cell line, while the transversion revealed was G-T in 1 cell

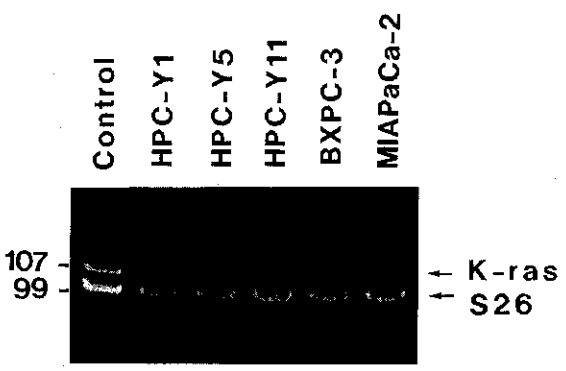

Fig. 2. Determination of the copy number of the K-*ras* gene. PCR was carried out with 100 ng of genomic DNA with primer sets for the K-*ras* and the S26 genes. The ratios of radioactivity of the K-*ras* gene to that of the S26 gene were similar among all the cell lines examined. Peripheral blood leukocytes from a normal person were used as a negative control.

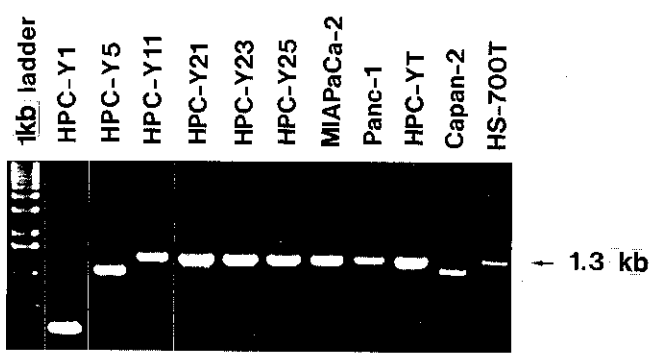

Fig. 3. Agarose gel electrophoresis of RT-PCR products of the p53 mRNA. Three cell lines, HPC-Y1, HPC-Y5 and Capan-2 show transcripts smaller than the expected size, 1.3 kb.

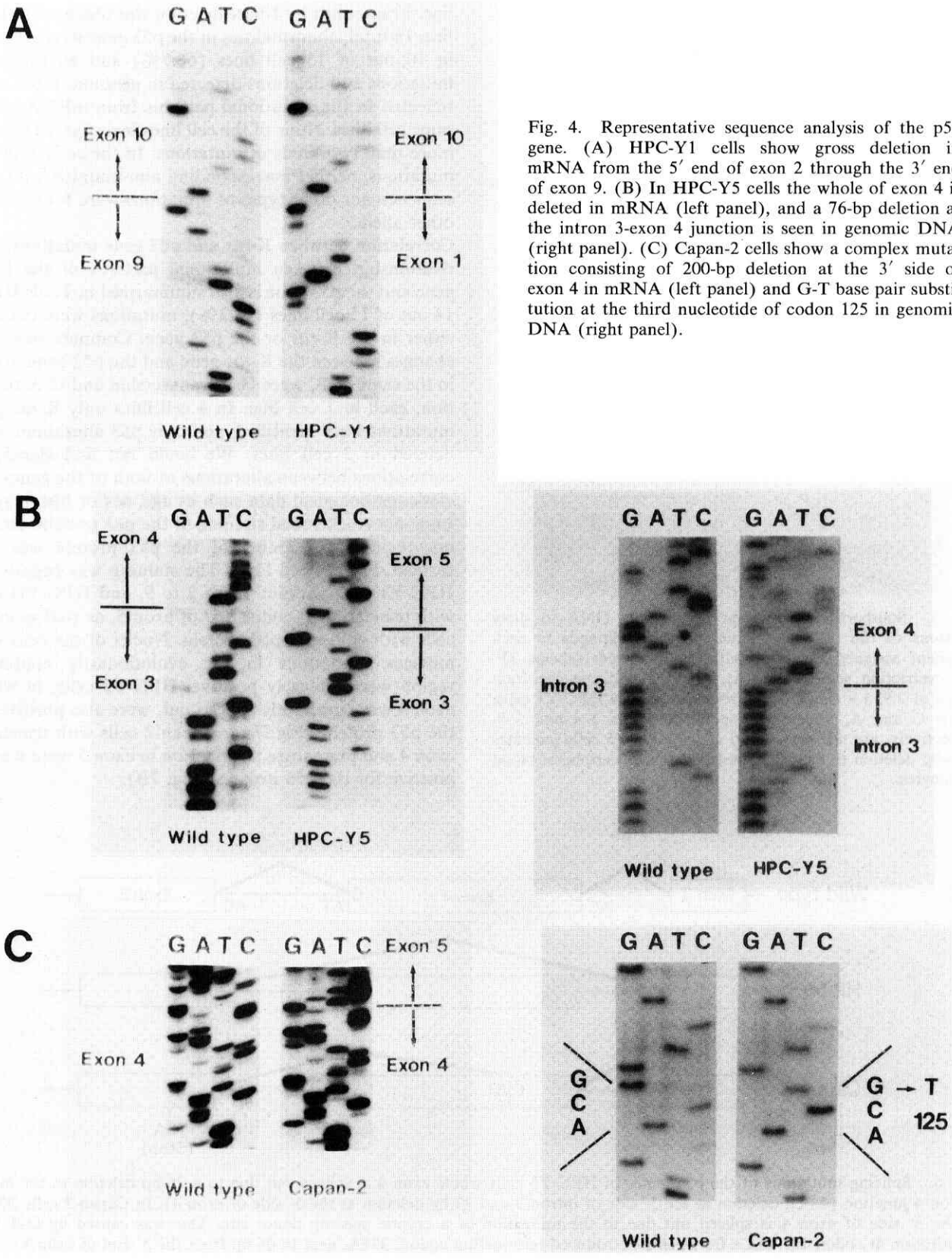

Fig. 4. Representative sequence analysis of the p53 gene. (A) HPC-Y1 cells show gross deletion in mRNA from the 5′ end of exon 2 through the 3′ end of exon 9. (B) In HPC-Y5 cells the whole of exon 4 is deleted in mRNA (left panel), and a 76-bp deletion at the intron 3-exon 4 junction is seen in genomic DNA (right panel). (C) Capan-2 cells show a complex mutation consisting of 200-bp deletion at the 3′ side of exon 4 in mRNA (left panel) and G-T base pair substitution at the third nucleotide of codon 125 in genomic DNA (right panel).

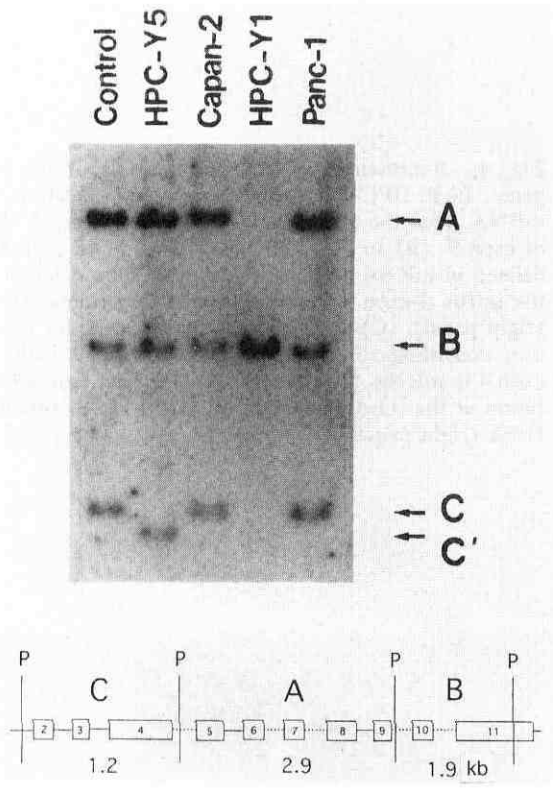

Fig. 5. Southern hybridization of genomic DNA to show deletions of the p53 gene. Each band corresponds to each fragment assigned the same letter in the lower schema (P: *Pst*I restriction sites. Bands A, B and C indicate the fragments of 2.9, 1.9 and 1.2 kbp, respectively). In HPC-Y1 cells, bands C and A, which correspond to exons 2–4 and 5–9, respectively, are not seen. Band C′ of HPC-Y5 cells indicates a 76-bp deletion in band C. Control: normal peripheral blood leukocytes.

line. Frame shift by 1-base deletion was observed in 1 cell line. In total, abnormalities in the p53 gene were detected in 10 out of 15 cell lines (66.7%) and all the point mutations and deletions detected in genomic DNA were reflected in the mutational patterns from mRNA of the same cell lines. None of the cell lines investigated showed more than two kinds of mutations. In the cells with p53 mutations, neither corresponding non-mutated wild type sequence nor other types of mutations were found in the other allele.

Correlation between K-*ras* and p53 gene mutations The relationship between mutational patterns of the K-*ras* gene and the p53 gene is also summarized in Table III. In 14 out of 15 cell lines (93.3%), mutations were detected either in the K-*ras* or the p53 gene. Common base pair changes between the K-*ras* gene and the p53 gene, found in the same cells, were G-T transversion and G-A transition, each in 1 cell line. In 4 cell lines only K-*ras* gene mutations were identified, and only p53 alterations were deteted in 3 cell lines. We could not find significant correlations between alterations of both of the genes and clinicopathological data such as age, sex or histology.

Immunocytochemical staining of the p53 protein Immunocytochemical staining of the p53 protein was performed with 10 cell lines. The staining was negative in HPC-Y1 cells without exons 2 to 9, and HPC-YO cells with truncation at codon 169 of exon 5, as well as in the cells with wild-type p53 protein. Nuclei of the cells with missense mutations in the evolutionarily conserved region were intensely positive. HPC-Y5 cells, in which exon 4 was completely spliced out, were also positive for the p53 protein (Fig. 7A). Capan-2 cells with truncated exon 4 and premature termination in exon 5 were weakly positive for the p53 protein (Fig. 7B).

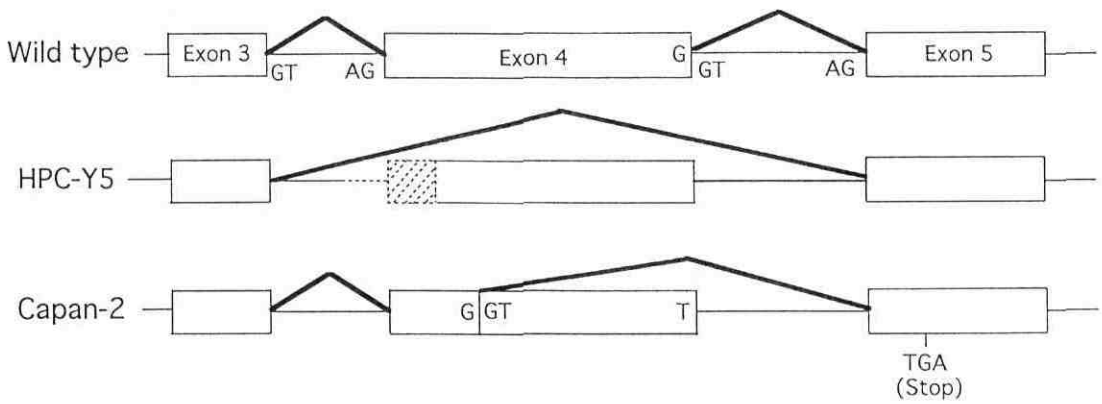

Fig. 6. Splicing mutations of the p53 gene. In HPC-Y5 cells whole exon 4 is spliced out due to a 76-bp deletion in the intron 3-exon 4 junction (43-bp deletion at the 3′ side of intron 3 and 33-bp deletion at the 5′ side of exon 4). In Capan-2 cells 200 bp of the 3′ side of exon 4 is spliced out due to the utilization of a cryptic splicing donor site. This was caused by G-T base substitution at codon 125 and a frame shift-introduced termination codon, TGA, next to 65 bp from the 5′ end of exon 5.

Table III. Mutations of the K-*ras* and p53 Genes

Cell line	K-*ras* mutation in codon 12[a]		p53 mutation				p53 staining[d]
	Nucleotide	Amino acid	Exon	Codon	Nucleotide	Amino acid	
HPC-Y1	GTT/WT[b]	Val	2-9	deletion of exons			−
HPC-Y3	GAT/WT	Asp	WT[b]				±
HPC-Y5	GAT/WT	Asp	4	76 bp deletion[c]		splicing mutation	+
HPC-Y11	TGT/GTT	Cys/Val	WT				−
HPC-Y19	WT		8	266	GGA→GAA	Gly→Glu	ND
HPC-Y21	GTT/WT	Val	6	193	CAT→CGT	His→Arg	ND
HPC-Y23	GAT/WT	Asp	WT				ND
HPC-Y25	GAT/WT	Asp	WT				ND
Capan-2	GTT/WT	Val	4	125	ACG→ACT	splicing mutation	±
HS-700T	WT		7	249	AGG→ATG	Arg→Met	ND
HPC-YO	GTT/WT	Val	5	152	CCG→-CG	Stop at 169	−
HPC-YT	WT		WT				−
BXPC-3	WT		6	220	TAT→TGT	Tyr→Cys	+
MIAPaCa-2	TGT	Cys	7	248	CGG→TGG	Arg→Trp	+
Panc-1	GAT/WT	Asp	8	273	CGT→CAT	Arg→His	+

a) All the K-*ras* mutations involved codon 12. *b*) Wild type. *c*) 76 bp deletion in intron 3-exon 4 junction.
d) +, positive; ±, weakly positive; −, negative; ND, not done.

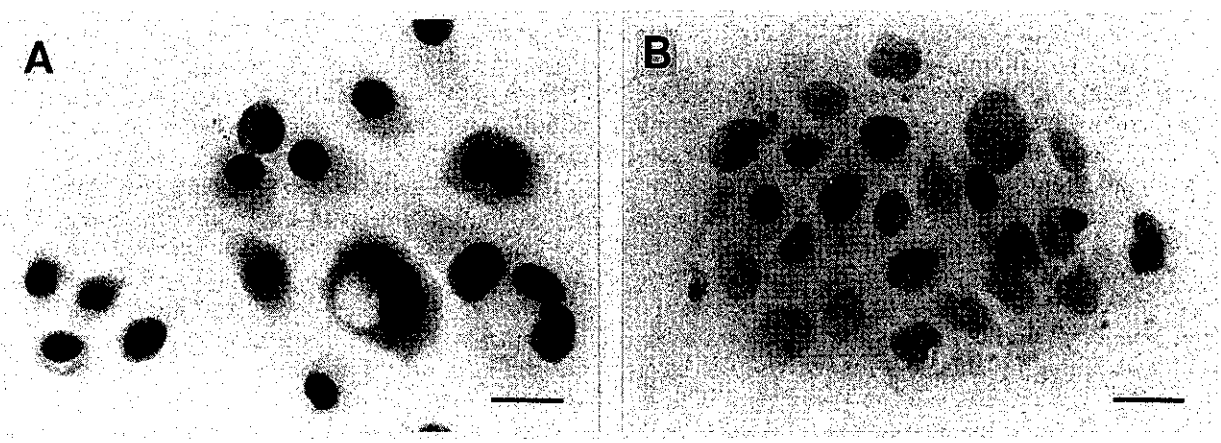

Fig. 7. Immunocytochemical staining of the p53 protein. Nuclei are intensively positive in HPC-Y5 cells (A) and are weakly positive in Capan-2 cells (B). Scale bars, 10 μm.

DISCUSSION

In the present study, we analyzed alterations of mRNA as well as mutations of the K-*ras* oncogene and the p53 tumor suppressor gene in 15 human pancreatic carcinoma cell lines. Furthermore immunocytochemical staining of the p53 protein in representative cell lines was performed to compare genetic changes and phenotypic characteristics.

The incidence of K-*ras* gene mutation in the present study was 73.3%, which is lower than in previous reports.[5-8] Considering that the cell lines established in the current study were biased to well-differentiated subtype, they may be a special set with a relatively low incidence of K-*ras* point mutation. Nine out of 11 cell lines with K-*ras* gene mutations concomitantly contained alleles of mutant and wild types, and both the alleles were expressed into mRNA. Although both alleles of codon 12 of the K-*ras* gene were mutated in HPC-Y11 cells, only the allele with TGT was expressed into mRNA while that with GTT was not. MIAPaCa-2 contained the only TGT sequence which was transcribed. Although the TGT sequence was a single copy per haploid, we do not know whether both alleles had mutated into TGT, or whether the TGT allele had been duplicated while the other allele had been deleted. Bos[23] reported that during progression

of a tumor the ratio between wild and mutant alleles can shift towards the mutant alleles. Amplification of the K-*ras* gene with and without point mutation has been reported. As far as we could determine, the amplified K-*ras* gene always comes from the mutated allele if the cells have both mutated and wild-type alleles.[24] Furthermore, TGT is almost always the detected point mutation in the amplified allele.[25-27] Expression of the mutated K-*ras* gene, especially with TGT at codon 12, may give a growth advantage to pancreatic carcinoma or interfere with the counterpart allele of the K-*ras* gene.

The frequency and distribution of hot spots of mutation of the p53 gene differ among cancers from different tissue types.[28] In the present study, alterations in the p53 gene were detected in 10 cell lines (66.7%), which is consistent with the report by Scarpa *et al.*[16] They and others reported 1-bp substitutions at codons 220, 249 and 273. To date, the 1-bp substitutions at codons 193 and 266, and 1-bp deletion at codon 152 found in the present study, have not been identified in pancreatic adenocarcinoma. Scarpa *et al.*[16] also reported deletion at codon 152 followed by the stop codon. In the present study, a frame shift caused by a deletion in codon 152 changed codon 169 to a TGA termination codon in HPC-YO cells, suggesting that codon 152 may be a hot spot susceptible to carcinogenic insult in the pancreas. A point mutation at the CpG dinucleotide site, which is frequently observed in colon carcinoma,[13] and which represents spontaneous mutation, was detected in only one cell line in the present study. The mutational patterns observed in the cell lines HS-700T, BXPC-3, MIAPaCa-2 and Panc-1 are in agreement with observations in previous studies on the same cell lines.[3, 14, 15] In the present study, G-T transversion, which is caused by chemical agents, was the most frequently observed mutation in the K-*ras* gene. This is consistent with the fact that smoking is assumed to be a carcinogen in the pancreas.[1, 2] However, G-T transversion was less frequent in the p53 gene. Furthermore, pancreatic carcinoma has not been conclusively identified as one of the component tumors in Li-Fraumeni syndrome, which is characterized by germ line p53 mutation.[29] These observations suggest that, although abnormalities of the K-*ras* and the p53 genes are cooperatively associated with oncogenesis of pancreatic adenocarcinoma, different carcinogenic insults are involved in the mutations of these genes.

Only a limited number of splicing mutations of the p53 gene have been documented (Table IV). In these reports, more than 80% of mutational sites are in introns, and G-T transversion at the splicing donor site is the most frequent. About 70% of splicing mutations affect the evolutionarily conserved part of the p53 protein. To the best of our knowledge, splicing mutations have not previously been reported in pancreatic carcinoma. Although altered size of mRNA in Capan-2 cells has been reported, splicing mutations have not been identified because only exons 5 to 8 of genomic DNA were sequenced.[15] The same mutation as was seen in Capan-2 cells in this study has been reported in a small cell lung cancer cell line,[33] suggesting that common carcinogenic mechanisms exist between these cancers. Interestingly, a gross deletion from exons 2 to 9 identified in HPC-Y1 cells is a novel alteration of the p53 gene which has never before been documented.

It has been reported that mutant p53 proteins at the evolutionarily conserved region are detectable immunocytochemically because of protein accumulation owing to prolongation of their half life compared with normal p53 protein.[38, 39] As the antibody DO-7 recognizes the amino terminus of the p53 protein, it could react with any variant form of p53 as long as it is translated. Immunocytochemically the p53 protein without exon 4 was positive and the protein with part of exon 4 and truncated within exon 5 was weakly positive. However, p53 protein

Table IV. Splicing Mutations of the p53 Gene in Various Human Malignancies

Cancer location[a]	Total mutation[b]	Splicing mutation[c]	Location and frequency of splicing mutation												Reference
			I[d]	3	4	5	6	7	8	9	E[e]	4	6	8	
SCLC	?	1/?						1 (d[f])							Sameshima *et al.*[30] (1990)
NSCLC	57/77	4/57 (0.07)				1 (a[g])	1 (d)	1 (d)	1 (d)						Mitsudomi *et al.*[31] (1992)
Oral cavity	12/15	2/12 (16.7)					1 (d)						1		Sakai and Tsuchida.[32] (1992)
SCLC	18/?	5/18 (27.8)		1 (a)			1 (a)	1 (d)		1 (d)		1			Bodner *et al.*[33] (1992)
Sarcoma	12/127	2/12 (16.7)							1 (a)	1 (d)					Toguchida *et al.*[34] (1992)
Ovary	31/38	2/31 (9.5)			1 (d)									1	Kupryjańzyk *et al.*[35] (1993)
Liver	?/45	4/?		1 (a)			2 (a)	1 (d)							Lai *et al.*[36] (1993)
Stomach	15/29	1/15 (6.7)											1		Renault *et al.*[37] (1993)
Pancreas	10/15	2/10 (20)		1 (a)								1			This study

a) SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma. *b*) Cases with mutation/total cases. *c*) Splicing mutation/total mutation (%). *d*) Intron. *e*) Exon-intron boundary. *f*) Splicing donor site. *g*) Splicing acceptor site.

with exon 4 but truncated in exon 5, and that without exons 2 to 9 were negative. It is possible that mutations outside the evolutionarily conserved regions can affect the stability and translational capacity of the p53 mRNA[12] or conformation of the p53 protein.

It has been suggested that K-*ras* alteration is an early event[40] whereas p53 mutation is related to progression of human pancreatic carcinoma.[41] However, our study showed that alterations in the p53 gene as well as point mutations in the K-*ras* gene are very frequent genetic changes and we suggest that the abnormalities of both genes represent accumulated genetic changes which can cooperatively play an important role in the carcinogenic process of pancreatic adenocarcinoma. In addition to analysis of genomic DNA, analysis of transcribed mRNA of both genes could offer significant information about the carcinogenic process.

ACKNOWLEDGMENTS

We would like to thank Mr. Toda for his technical assistance, Dr. Miwa for generously providing a p53 cDNA probe, and Dr. Kaufman for her pertinent discussion.

(Received May 9, 1994/Accepted July 26, 1994)

REFERENCES

1) Poston, G. J., Gillespie, J. and Guillou, P. J. Biology of pancreatic cancer. *Gut,* **32,** 800–812 (1991).
2) Warshaw, A. L. and Castillo, C. F. Pancreatic carcinoma. *N. Engl. J. Med.,* **326,** 455–465 (1992).
3) Ruggeri, B., Zhang, S.-Y., Caamano, J., Dirado, M., Flynn, S. D. and Klein-Szanto, A. J. P. Human pancreatic carcinomas and cell lines reveal frequent and multiple alterations in the p53 and Rb-1 tumor-suppressor genes. *Oncogene,* **7,** 1503–1511 (1992).
4) Bishop, J. M. Molecular themes in oncogenesis. *Cell,* **64,** 235–248 (1991).
5) Hruban, R. H., Van Mansfeld, A. D. M., Offerhaus, G. J. A., Van Weering, D. H. J., Allison, D. C., Goodman, S. N., Kensler, T. W., Bose, K. K., Cameron, J. and Bos, J. L. K-*ras* oncogene activation in adenocarcinoma of the human pancreas. *Am. J. Pathol.,* **143,** 545–554 (1993).
6) Almoguera, C., Shibata, D., Forrester, K., Martin, J. and Perucho, M. Most human carcinoma of the exocrine pancreas contain mutant c-K-*ras* genes. *Cell,* **53,** 549–554 (1988).
7) Smit, V, T., Boot, A, J., Smits, A. A., Fleuren, G. J., Cornelisse, C. J. and Bos, J. L. K-*ras* codon 12 mutations occur very frequently in pancreatic adenocarcinoma. *Nucleic Acids Res.,* **16,** 7773–7782 (1988).
8) Nagata, Y., Abe, M., Motoshima, K., Nakayama, E. and Shiku, H. Frequent glycine-to-aspartic acid mutations at codon 12 of c-Ki-*ras* gene in human pancreatic cancer in Japanese. *Jpn. J. Cancer Res.,* **81,** 135–140 (1990).
9) Cerny, W. L., Mangold, K. A. and Scarpelli, D. G. K-*ras* mutation is an early event in pancreatic duct carcinogenesis in the Syrian golden hamster. *Cancer Res.,* **52,** 4507–4513 (1992).
10) Tsutsumi, M., Kondoh, S., Noguchi, O., Horiguchi, K., Kobayashi, E., Okita, S., Ohashi, K., Honoki, K., Tsujiuchi, T. and Konishi, Y. K-*ras* gene mutation in early ductal lesions induced in a rapid production model for pancreatic carcinomas in Syrian hamsters. *Jpn. J. Cancer Res.,* **84,** 1101–1105 (1993).
11) Shibata, D., Capella, G. and Perucho, M. Mutational activation of the c-K-*ras* gene in human pancreatic carcinoma. *Baillieres Clin. Gastroenterol.,* **4,** 151–169 (1990).
12) Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Clesry, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C. and Vogelstein, B. Mutations in the p53 gene occur in diverse human tumour types. *Nature,* **342,** 705–708 (1989).
13) Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. C. p53 mutations in human cancers. *Science,* **253,** 49–52 (1991).
14) Barton, C. M., Staddon, S. L., Hughes, C. M., Hall, P. A., O'Sullivan, C., Kloppel, G., Theis, B., Russell, R. C. G., Neoptolemos, J., Williamson, R. C. N., Lane, D. P. and Lemoine, N. R. Abnormalities of the p53 tumor suppressor gene in human pancreatic cancer. *Br. J. Cancer,* **64,** 1076–1082 (1991).
15) Kalthoff, H., Schmiegel, W., Roeder, C., Kasche, D., Schmidt, A., Lauer, G., Thiele, H. G., Honold, G., Pantel, K., Riethmuller, G., Scherer, E., Maurer, J., Maacke, H. and Deppert, W. p53 and K-*RAS* alterations in pancreatic epithelial cell lesions. *Oncogene,* **8,** 289–298 (1993).
16) Scarpa, A., Capelli, P., Mukai, K., Zamboni, G., Oda, T., Lacono, C. and Hirohashi, S. Pancreatic adenocarcinoma frequently show p53 gene mutations. *Am. J. Pathol.,* **142,** 1534–1543 (1993).
17) Yamaguchi, N., Yamamura, Y., Koyama, K., Ohtsuji, E., Imanishi, J. and Ashihara, T. Characterization of new human pancreatic cancer cell lines which propagate in a protein-free chemically defined medium. *Cancer Res.,* **50,** 7008–7014 (1990).
18) Fukumoto, M., Estensen, R., Sha, L., Oakley, G. J., Twiggs, L. B., Adcock, L. L., Carson, L. F. and Roninson, I. B. Association of Ki-*ras* with amplified DNA sequences, detected in human ovarian carcinomas by a modified in-gel renaturation assay. *Cacner Res.,* **49,** 1693–1697 (1988).
19) Arao, S., Suwa, H., Mandai, M., Tashiro, H., Miyazaki, K., Okamura, H., Nomura, H., Hiai, H. and Fukumoto, M. Expression of multidrug resistance gene and localization of P-glycoprotein in human primary ovarian cancer. *Cancer Res.,* **54,** 1355–1359 (1994).

20) Vincent, S., Marty, L. and Fort, P. S26 ribosomal protein RNA: an invariant control for gene regulation experiments in eucaryotic cells and tissues. *Nucleic Acids Res.*, **21,** 1498 (1993).
21) Miwa, H., Kita, K., Saya, H., Ohtsuji, A., Masuya, M., Nishii, K., Morita, N., Takakura, N., Ohishi, K., Nasu, K., Nakase, K. and Shirakawa, S. Structural alterations of the p53 gene in human leukemias. *Leuk. Res.*, **16,** 1105–1112 (1992).
22) Vojtěšek, B., Bártek, J., Midgley, C. A. and Lane, D. P. An immunohistochemical analysis of the human nuclear phosphoprotein p53. *J. Immunol. Methods,* **151,** 237–244 (1992).
23) Bos, J. L. The *ras* gene family and human carcinogenesis. *Mutat. Res.*, **195,** 255–271 (1988).
24) Shiraishi, M., Noguchi, M., Shimosato, Y. and Sekiya, T. Amplification of protooncogenes in surgical specimen of human lung carcinomas. *Cancer Res.*, **49,** 6474–6479 (1989).
25) Taya, Y., Hosogai, K., Hirohashi, S., Shimosato, Y., Tsuchiya, R., Tsuchida, N., Fushimi, M., Sekiya, T. and Nishimura, S. A novel combination of K-*ras* and *myc* amplification accompanied by point mutational activation of K-*ras* in a human lung cancer. *EMBO J.*, **3,** 2943–2946 (1984).
26) Winter, E., Yamamoto, F., Almoguera, C. and Perucho, M. A method to detect and characterize point mutations in transcribed genes: amplification and overexpression of the mutant c-Ki-*ras* allele in human tumor cells. *Proc. Natl. Acad. Sci. USA,* **82,** 7575–7579 (1985).
27) Slebos, R. J. C., Habets, G. G. M., Evers, S. G., Mooi, W. J. and Rodenhuis, S. Allele-specific detection of K-*ras* oncogene expression in human non-small-cell lung carcinomas. *Int. J. Cancer,* **48,** 51–56 (1991).
28) Levine, A. J., Momand, J. and Finlay, C. A. The p53 tumor suppressor gene. *Nature,* **351,** 453–456 (1991).
29) Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Nelson, C. E., Kim, D. H., Kassel, J., Gryka, M. A., Bischoff, F. Z., Tainsky, M. A. and Freind, S. H. Germ line p53 mutations in a familial syndrome of breast cancer, sarcoma and other neoplasms. *Science,* **250,** 1233–1238 (1990).
30) Sameshima, Y., Akiyama, T., Mori, N., Mizoguchi, H., Toyoshima, K., Sugimura, T., Terada, M. and Yokota, J. Point mutation of the p53 gene resulting in splicing inhibition in small cell lung carcinoma. *Biochem. Biophys. Res. Commun.*, **173,** 697–703 (1990).
31) Mitsudomi, T., Steinberg, S. M., Nau, M. M., Carbone, D., D'Amico, D., Bodner, S., Oie, H. K., Linnoila, R. I., Mulshine, J. L., Minna, J. D. and Gazder, A. F. p53 gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of *ras* mutations and clinical features. *Oncogene,* **7,** 171–180 (1992).
32) Sakai, E. and Tsuchida, N. Most human squamous cell carcinoma in the oral cavity contain mutated p53 tumor-suppressor genes. *Oncogene,* **7,** 927–933 (1992).
33) Bodner, S. M., Minna, J. D., Jensen, S. M., D'Amico, D., Carbone, D., Mitsudomi, T., Fedorko, J., Buchhagen, D. L., Nau, M. M., Gazdar, A. F. and Linnoila, R. I. Expression of mutant p53 proteins in lung cancer correlates with the class of p53 gene mutations. *Oncogene,* **7,** 743–749 (1992).
34) Toguchida, J., Yamaguchi, T., Ritchie, B., Beauchamp, R. L., Dayton, S. H., Herrera, G. E., Yamamuro, T., Kotoura, Y., Sasaki, M. S., Little, J. B., Weichselbaum, R. R., Ishizaki, K. and Yandell, D. W. Mutation spectrum of the p53 gene in bone and soft tissue sarcoma. *Cancer Res.*, **52,** 6194–6199 (1992).
35) Kupryjańzyk, J., Thor, A. N., Beauchamp, R., Merritt, V., Edgerton, S. M., Bell, D. A. and Yandell, D. W. p53 gene mutations and protein accumulation in human ovarian cancer. *Proc. Natl. Acad. Sci. USA,* **90,** 4961–4965 (1993).
36) Lai, M. Y., Chang, H. C., Li, H. P., Ku, C. K., Chen, P. J., Sheu, J. C., Huang, G. T., Lee, P. H. and Chen, D. S. Splicing mutations of the p53 gene in human hepatocellular carcinoma. *Cancer Res.*, **53,** 1653–1656 (1993).
37) Renault, B., Van Den Broek, M., Fodde, R., Wijnen, J., Pellegata, N. S., Amadori, D., Khan, P. M. and Ranzani, G. N. Base transitions are the most frequent genetic changes at p53 in gastric cancer. *Cancer Res.*, **53,** 2614–2617 (1993).
38) Iggo, R., Gatter, K., Barter, J., Lane, D. and Harris, A. L. Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet,* **335,** 675–679 (1990).
39) Shaulsky, G., Goldfinger, N., Ben-ze'ev, A. and Rotter, V. Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis. *Mol. Cell. Biol.*, **10,** 6565–6577 (1990).
40) Yanagisawa, A., Ohtake, K., Ohashi, K., Hori, M., Kitagawa, T., Sugano, H. and Kato, Y. Frequent c-Ki-*ras* oncogene activation in mucous cell hyperplasias of pancreas suffering from chronic inflammation. *Cancer Res.*, **53,** 953–956 (1993).
41) Pellegata, N. S., Sessa, F., Renault, B., Bonato, M., Leone, B. E., Solcia, E. and Ranzani, G. N. K-*ras* and p53 mutations in pancreatic cancer: ductal and nonductal tumors progress through different genetic lesions. *Cancer Res.*, **54,** 1556–1560 (1994).