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K-*ras* and *p53* Gene Mutations in Pancreatic Cancer: Ductal and Nonductal Tumors Progress through Different Genetic Lesions[1]

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ABSTRACT

We studied K-*ras* and *p53* gene mutations in a panel of 57 primary pancreatic cancers including ductal and nonductal tumors. DNAs were obtained from formalin-fixed, paraffin-embedded material. Target sequences were amplified by polymerase chain reaction and analyzed by denaturing gradient gel electrophoresis and sequencing. Both K-*ras* and *p53* genes were frequently mutated in ductal cancers (25 of 35, 71.4%; 18 of 35, 51.4%, respectively). K-*ras* mutations were confined to the second position of codon 12 where base transitions and transversions were equally observed. *p53* changes were mainly missense mutations. Transitions and transversions were found equally with a prevalence of G:C→A:T changes among transitions. No gene alterations were present in the 6 exocrine nonductal tumors and (with one exception) in the 12 endocrine tumors analyzed. Our results indicate that mutated K-*ras* and *p53* genes can cooperate in the establishment of ductal pancreatic cancers, whereas other genetic events have to be present in nonductal tumors. Moreover, K-*ras* alterations may represent an early event in ductal tumorigenesis, as suggested both by the high gene mutation frequency and by the presence of mutations in low-grade tumors. On the contrary, *p53* gene changes seem to represent an event required for the malignancy progression of ductal tumors from lower to higher grades.

INTRODUCTION

Ductal tumor of the exocrine pancreas is the most common (about 90%) and well-studied pancreatic tumor type. It represents the fourth leading cause of death from malignant diseases in Western countries and its incidence appears to be increasing (1, 2). The aggressive nature of the neoplasia, the lack of early detection, and the refractory response to the treatments contribute to the high mortality rate from the disease.

Progress in understanding the molecular pathology of pancreatic neoplasias has been restrained by the limited availability of biological material. In spite of this fact, genetic abnormalities have been described, involving growth factors and their receptors (3–6), as well as oncogenes (7–11) and tumor suppressor genes (12–17).

Point mutations of the K-*ras* protooncogene, almost always confined to codon 12, are the most frequent genetic changes (about 80%) and may represent a relatively early event in pancreatic tumorigenesis (7, 8, 18). Abnormally high levels of *p53* gene product have been found in 60% of pancreatic tumors (12, 16), suggesting a high mutation frequency of the gene in this neoplasia. In fact, a high *p53* expression level results in most cases from a longer half-life of the protein associated with point mutations within the coding part of the gene (19). More recently, both K-*ras* and *p53* alterations have been identified in pancreatic cell lines, suggesting a cooperation effect which may contribute to the malignant phenotype (15).

We studied K-*ras* and *p53* mutations in a panel of 57 primary pancreatic tumors. The sample included tumor types related to the different cell compartments of the pancreas, *i.e.*, acinar, ductal, and endocrine cells. Our aim was to determine the gene mutation frequencies and to evaluate the cooperative effect of K-*ras* and *p53* alterations in pancreatic tumors of different types and with various degrees of anaplasia. We were also interested in determining the *p53* mutation pattern in pancreatic cancer. Although *p53* mutations are the most common genetic changes in human cancer, their incidence, distribution, and nature show cancer type and tissue specificity (20, 21). Analysis of the gene can, therefore, provide some clues concerning both endogenous cellular mechanisms and exogenous agents related to mutagenic processes acting in different tissues.

Tumor sample DNAs were obtained from formalin-fixed, paraffin-embedded material and analyzed by PCR[3]-based DGGE and sequencing methods.

MATERIALS AND METHODS

Tissue Sampling and Morphology. Surgical specimens of 57 pancreatic tumors were collected from the files of the Pathology Departments of the Istituto di Ricovero e Cura a Carattere Scientifico-Ospedale S. Matteo of Pavia, Ospedale di Circolo of Varese, and Istituto di Ricovero e Cura a Carattere Scientifico-Ospedale S. Raffaele of Milan. Two or three representative formalin-fixed, paraffin-embedded blocks from each tumor were available for histological investigation.

The tumors were classified histologically according to the criteria of Cubilla and Fitzgerald (22). The sample included 35 ductal carcinomas (all with metastases) and 18 nonductal tumors (12 clinically nonfunctioning large endocrine tumors, 8 of which with malignancy proven by metastases and 4 with moderate histological atypia but no metastases; 3 acinar cell carcinomas; 1 papillary cystic tumor; 1 serous cystadenoma; and 1 ductuloacinar carcinoma). Two mucinous cystadenocarcinomas and 2 giant cell carcinomas were also examined. Mucinous cystadenocarcinoma and giant cell carcinoma are interpreted as low- and high-grade growths, respectively, of the same ductal cell involved in the histogenesis of ductal carcinoma.

Grading of ductal carcinomas (13 G1, 15 G2, and 7 G3 tumors) was done according to the criteria of Kloppel (23).

We characterized exocrine tumors with immunoperoxidase by using antibodies against pancreatic and gastrointestinal mucin antigens (24). Monoclonal antibody anti-human lipase (Chemicon, Inc., Temecula, CA) and anti-human trypsinogen (Biodesign Int., Kennebunkport, ME) were used to characterize acinar cell carcinomas. None of the 12 endocrine tumors studied was associated with a hyperfunctional syndrome. Such clinically "nonfunctioning" endocrine tumors were investigated with Grimelius' silver and by immunohistochemical staining for chromogranin A, insulin, glucagon, somatostatin, pancreatic polypeptide, and gastrin as previously reported (25). All tumors gave widespread staining with general endocrine markers. No hormone immunoreactivity was detected in two of the malignant cases, while in the remaining tumors minority populations of glucagon (6 cases), pancreatic polypeptide (6), insulin (3), gastrin (2), or somatostatin (2) cells were detected.

DNA Preparation. Sections (5 μm) of formalin-fixed, paraffin-embedded tumor tissue sections were dissected following closely the distribution of tumor tissue in order to ensure that >70% of the remaining section was formed by tumor. Such sections were incubated overnight at 58°C in 200 μl of extraction

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[3] The abbreviations used are: PCR, polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Glu, glutamic acid; Gly, glycine; His, histidine; Leu, leucine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

buffer (50 mM KCl-10 mM Tris-HCl, pH 7.5-2.5 mM $MgCl_2$-0.1 mg/ml gelatin-0.45% Nonidet P-40-0.45% Tween-20-500 μg/ml proteinase K). The solution was heated for 15 min at 95°C to inactivate the proteinase K, and then it was centrifuged. The supernatant (5–10 μl) was used directly for PCR amplification.

PCR Amplification. The DNA extracted from tissue sections was a suitable template for the amplification of the relatively short target DNA fragments (111–270 base pairs). The sequences of the specific primers utilized for K-*ras* and for *p53* gene amplification are those we previously reported (Refs. 26 and 27, respectively). One member of each primer pair contained a 5′ 40-base pair GC-rich sequence (GC-clamp). GC-clamped primers were used to obtain amplified products suitable for DGGE analysis.

Amplification was performed on 5–10 μl of DNA by using 12.5 pmol of each primer, 1 unit of thermostable DNA polymerase, and 200 μM deoxynucleoside triphosphates in a volume of 50 μl of buffer (50 mM KCl-10 mM Tris-HCl, pH 8.4-2 mM $MgCl_2$-0.2 mg/ml of bovine serum albumin). Thirty to 40 cycles of amplification were performed in a programmable thermal cycler (MJ Research, Inc.). One cycle consisted of: (*a*) 1 min at 94°C; (*b*) 1.5 min at 55°C for K-*ras* gene exon 1, at 53°C for exons 5A, 5B, and 6 of *p53* gene, at 56°C for exon 7, and at 58°C for exon 8; (*c*) 2 min at 72°C.

DGGE. The PCR products obtained with the GC-clamped ampliprimers were analyzed by DGGE. The introduction of a GC-clamp into the amplified fragments brings the probability of detecting any base change within the fragment close to 100% (28).

The gel apparatus was essentially as described by Myers *et al.* (29). The appropriate DGGE conditions for the analysis of K-*ras* gene exon 1 (26) and for *p53* gene exons 5–8 (27) were as we previously reported.

In our hands (26) the sensitivity of the PCR-based DGGE method allows the detection of an heterozygous mutation present in 4% of the cells utilized for DNA extraction. Samples showing a variant DGGE pattern were reamplified and reanalyzed by DGGE in independent experiments.

DNA Sequencing. Genomic DNAs from samples showing variant DGGE patterns were amplified using the appropriate primers without GC-clamps in a total volume of 100 μl.

The amplification product was purified on a 2% low-melting agarose gel. The specific band was excised from the gel, frozen in liquid nitrogen for a few minutes, and incubated for 15 min at 37°C. This step was repeated 2 or 3 times to recover all of the DNA. The agarose fragment was centrifuged for 10 min, and the supernatant was quantified and directly utilized for sequencing reactions.

Sequencing was performed by the dideoxy procedure (30) using the Circum Vent(exo-) kit (New England Biolabs, Beverly, MA) for cyclic sequencing. Following the manufacturer's instructions, 10 ng of the template DNA were mixed with 1.2 pmol of the primer, 2 units of Vent(exo-) polymerase, and 2 μl of α-^{35}S-dATP in 16 μl of buffer [10 mM KCl-10 mM $(NH_4)_2SO_4$-20 mM Tris-HCl, pH 8.8-5 mM $MgSO_4$-0.2% Triton X-100]. Twenty cycles of amplification were performed in a programmable thermal cycler (MJ Research, Inc.), each cycle consisting of 20 s at 95°C, 20 s at the annealing temperature specific for the primer utilized, and 20 s at 70°C. PCR reactions were electrophoresed on 7% polyacrylamide-7 M urea gels for 2–3 h (1800 V). The gels were then washed for 15 min in 10% methanol and 10% acetic acid. Gels were dried and exposed to β-MAX films (Amersham).

RESULTS

We searched for K-*ras* and *p53* gene mutations in 57 pancreatic tumors by analyzing DNA from formalin-fixed, paraffin-embedded tissue samples. The screening was performed by using PCR, DGGE, and sequencing methods.

K-*ras* Gene. The first exon of the K-*ras* gene was amplified with specific ampliprimers, and the PCR product was analyzed by DGGE as we previously reported (26). Tumor samples were analyzed in parallel with DNAs from control cell lines homozygous for the wild-type gene or heterozygous for known mutated K-*ras* alleles. As mutated cell lines we chose those carrying K-*ras* base substitutions expected to occur frequently in pancreatic carcinomas. Tumor samples showing variant DGGE patterns were classified by simply comparing their DGGE bands with those of control cell lines as previously reported (26). The few samples showing bands different from those of control DNAs were sequenced in order to characterize the K-*ras* mutations.

All tumor samples carrying a mutated K-*ras* allele showed four DGGE bands, including the homoduplex one corresponding to the wild-type gene. This indicates that the neoplastic cells were heterozygous for the observed mutation and/or that nonneoplastic cells contributing the normal allele were present in the tissue sections utilized for DNA extraction. Fig. 1 shows patterns from tumors homozygous (*sample 7*) and heterozygous (*samples 2* and *3*) for normal and mutated K-*ras* alleles, respectively.

We observed K-*ras* gene mutations in 25 of 35 (71.4%) ductal pancreatic tumors. On the contrary, no mutations were observed in the remaining 22 cases, including 2 mucinous cystadenocarcinomas, 2 giant cell carcinomas, 6 nonductal exocrine tumors, and 12 nonfunctioning endocrine tumors.

All mutations occurred at codon 12, with the exception of one case (patient 29) showing a mutation at codon 13. Of these mutations, 21 (13 G→A and 8 G→T) occurred at the second position of the codon. One ductal tumor (patient 10) showed two different mutations at codon 12, namely, GGT→TGT (Gly→Cys) and GGT→AGT (Gly→Ser). All of the K-*ras* base changes observed are reported in the last column of Table 1.

***p53* Gene.** Exons 5–8 of the *p53* gene were amplified with specific ampliprimers, and the PCR products were analyzed by DGGE as we previously reported (27). The exons analyzed contain codons of most of the evolutionary conserved amino acids of p53 protein and include several mutation hot spots (20).

We found 20 tumors with a mutated *p53* sequence. All of the mutated samples showed four bands including the homoduplex one corresponding to the wild-type *p53* allele (Fig. 2).

In total we observed 14 different variant patterns, since few samples shared the same DGGE bands. Samples showing variant DGGE patterns were sequenced, and both sense and antisense strands were analyzed to confirm the nature of mutations. In all of the samples showing a variant pattern, DNA sequencing revealed the presence of a mutation in the coding part of the gene. The presence of the same base substitution was found in samples showing identical DGGE bands. The variant patterns were due to 3 different mutations localized in exon 5, to 1 in exon 6, to 5 in exon 7, and to 5 in exon 8 (Table 1).

Of 20 mutations, 18 were found in ductal carcinomas (18 of 35, 51.4%), whereas only one of 18 nonductal exocrine or endocrine

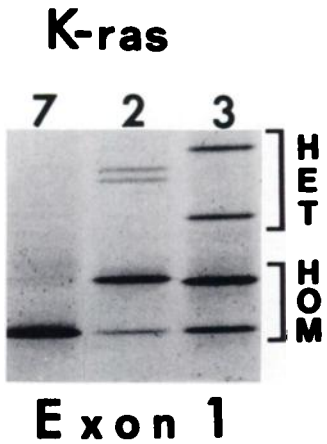

Fig. 1. Negative image of ethidium bromide-stained denaturing gradient gel showing patterns from 3 different samples. Tumor DNAs were amplified with 5′- and 3′-GC ampliprimers specific for the first exon of the K-*ras* gene, and the PCR products were loaded onto a 45–75% gradient of denaturants. *Sample 7*, normal sequence; *sample 2*, GGT→GAT mutation at codon 12 (Gly→Asp); *sample 3*, GGT→GTT mutation at codon 12 (Gly→Val). HET, heteroduplexes; HOM, homoduplexes.

Table 1 *K-ras and p53 mutations in pancreatic tumors*

Patient	Histological type	Histological grade	*p53* mutations				K-*ras* mutations at codon 12 (GGT = Gly)
			Exon	Codon	Base change	Amino acid substitution	
			Ductal tumors				
1	Ductal carcinoma	G1					GTT = Val
2	Ductal carcinoma	G2					GAT = Asp
3	Ductal carcinoma	G2	7	249	AGG → ACG	Arg → Thr	GTT = Val
4	Ductal carcinoma	G3	7	248	CGG → TGG	Arg → Trp	GAT = Asp
5	Ductal carcinoma	G3	8	273	CGT → TGT	Arg → Cys	GAT = Asp
6	Ductal carcinoma	G1					GAT = Asp
7	Ductal carcinoma	G1	8	282	CGG → TGG	Arg → Trp	
8	Ductal carcinoma	G2					
9	Ductal carcinoma	G3	8	298	GAG → TAG	Glu → stop	
10	Ductal carcinoma	G2					AGT, TGT = Ser/Cys
11	Ductal carcinoma	G3	8	273	CGT → TGT	Arg → Cys	
12	Ductal carcinoma	G1					
13	Ductal carcinoma	G1					GAT = Asp
14	Ductal carcinoma	G3	6	189	GCC → GGC	Ala → Gly	GAT = Asp
15	Ductal carcinoma	G1					
16	Ductal carcinoma	G3	8	273	CGT → TGT	Arg → Cys	GAT = Asp
17	Ductal carcinoma	G1	7	250	CCC → TCC	Pro → Ser	GAT = Asp
18	Ductal carcinoma	G1	8	273	CGT → CAT	Arg → His	
19	Ductal carcinoma	G2					GTT = Val
20	Ductal carcinoma	G1					CGT = Arg
21	Ductal carcinoma	G1	7	257	CTG → GTG	Leu → Val	CGT = Arg
22	Ductal carcinoma	G2	7	257	CTG → GTG	Leu → Val	GAT = Asp
23	Ductal carcinoma	G2	8	273	CGT → TGT	Arg → Cys	
24	Ductal carcinoma	G1					GAT = Asp
25	Ductal carcinoma	G3	5	131	AAC → CAC	Asn → His	GTT = Val
26	Ductal carcinoma	G1					
27	Ductal carcinoma	G2					GAT = Asp
28	Ductal carcinoma	G1					
29	Ductal carcinoma	G2	5	179	CAT → CGT	His → Arg	TGC[a] = Cys
30	Ductal carcinoma	G2	8	278	CCT → ACT	Pro → Thr	GTT = Val
31	Ductal carcinoma	G2	8	282	CGG → TGG	Arg → Trp	GAT = Asp
32	Ductal carcinoma	G2					GTT = Val
33	Ductal carcinoma	G2					GTT = Val
34	Ductal carcinoma	G2					GAT = Asp
35	Ductal carcinoma	G2	8	298	GAG → TAG	Glu → stop	GTT = Val
36	Mucinous cystadenocarcinoma						
37	Mucinous cystadenocarcinoma						
38	Giant cell carcinoma						
39	Giant cell carcinoma		7	255/256	TCA deleted	Ile missing	
			Nonductal tumors				
	Nonductal exocrine tumors						
40	Papillary-cystic tumor						
41	Serous cystadenoma						
42	Acinar cell carcinoma						
43	Acinar cell carcinoma						
44	Acinar cell carcinoma						
45	Ductuloacinar carcinoma						
	Nonfunctioning endocrine tumors						
46	Malignant						
47	Malignant						
48	Malignant						
49	Malignant						
50	Malignant						
51	Malignant						
52	Malignant						
53	Malignant						
54	Histology atypical						
55	Histology atypical		5	163	TAC → TGC	Tyr → Cys	
56	Histology atypical						
57	Histology atypical						

[a] Codon 13.

tumors (namely, a nonfunctioning endocrine tumor with moderate histological atypia but no metastases) showed a *p53* base change. Another mutation was found in a giant cell carcinoma, a type of tumor interpreted as a growth of anaplastic ductal cells.

Of 20 gene alterations, 17 were missense mutations. Two samples (patients 9 and 35) showed the same nonsense mutation in exon 8 and one tumor (patient 39) showed an in frame deletion of 3 base pairs. Sample 9, in addition to a functional mutation in exon 8, showed a silent nucleotide substitution in exon 6 (Fig. 2). This is a polymorphic variant already found in different population samples (31). Of the 19 point mutations, 11 were base transitions, 9 of them being G:C→A:T. All *p53* mutations are reported in Table 1, and specific base changes are summarized in Table 2.

K-*ras*, *p53* mutations and tumor grading of ductal carcinomas are summarized in Table 3.

DISCUSSION

By PCR-based DGGE and sequencing methods we analyzed K-*ras* and *p53* gene mutations in a panel of 57 pancreatic tumors of different

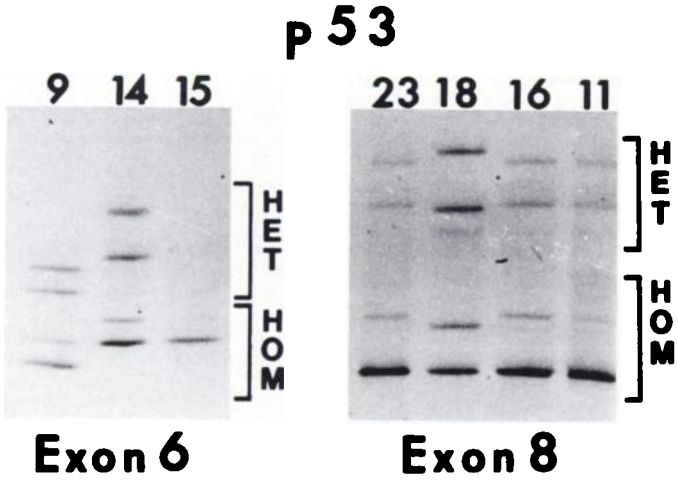

Fig. 2. Negative image of ethidium bromide-stained denaturing gradient gel showing patterns from different samples. DNAs were amplified with ampliprimers specific for exon 6 and exon 8 of the *p53* gene, and the PCR products were loaded onto a 50–70% and a 50–75% gradient of denaturants, respectively. Exon 6 (*left*): *sample 9*, silent CGA→CGG mutation at codon 213 (Arg→Arg); *sample 14*, GCC→GGC substitution at codon 189 (Ala→Gly); *sample 15*, normal sequence. Exon 8 (*right*): *samples 23, 11*, and *16*, same DGGE variant pattern as well as the same CGT→TGT substitution at codon 273 (Arg→Cys); *sample 18*, CGT→CAT mutation at codon 273 (Arg→His). HET, heteroduplexes; HOM, homoduplexes.

Table 2 *p53* point mutations in pancreatic tumors

	To[a]				
From	C	T	G	A	Total
C		8	3	1	12
T	0		0	0	0
G	1	2		1	4
A	1	0	2		3
Total	2	10	5	2	19

[a] Listed in coding strand.

types. The use of archival samples for DNA extraction allowed us to examine a relatively large number of ductal adenocarcinomas as well as to select rare tumor types among nonductal exocrine tumors, including serous cystadenoma, papillary-cystic tumor, acinar cell carcinoma, and ductuloacinar carcinoma. In addition, endocrine tumors of proven or suspected malignancy were investigated.

Our data confirm the remarkably high frequency of K-*ras* mutations in ductal carcinomas: we found 25 mutated tumors in 35 cases investigated, which corresponds to a frequency of 71.4%. As expected, the mutations were almost exclusively confined to the second position of codon 12 (21 of 25). We observed 14 of 25 base transitions (G→A) and 11 of 25 transversions (9 G→T and 2 G→C). These substitutions correspond to conversions from glycine to aspartic acid, valine, and arginine, respectively.

One sample (patient 10) showed two different mutations at the same position of codon 12. Because of the approach we used, we could not discriminate between the presence of two different mutations in the same tumor cells and the existence of two subpopulations of cells each carrying a single K-*ras* mutation. This last possibility would be in agreement with the finding by Grunewald *et al.* (32) of different K-*ras* mutations in different tissue areas within the same pancreatic tumors.

During the present survey we analyzed ductal tumors with different degrees of anaplasia. The presence of K-*ras* mutations also in low-grade G1 tumors (7 of 13, Table 3), in addition to the high mutation frequency found in ductal cancers in general, suggests that these genetic alterations may have a crucial role in ductal tumorigenesis. K-*ras* mutations also have been detected (33, 34) in intraductal papillary neoplasms, which are low-grade malignancies considered as possible precursors of ductal carcinoma, and even in some mucous cell hyperplasias of pancreases with chronic inflammation (35). These findings further support the idea (7, 8, 18) that K-*ras* mutations are an early event in ductal pancreatic cancerogenesis.

On the other hand, our data clearly demonstrate that K-*ras* mutations are not involved in the genesis and progression of nonductal exocrine or endocrine tumors. In fact, no such mutations were found in the 18 cases we analyzed. In addition, only one of 16 acinar cell carcinomas investigated by Hoorens *et al.* (17) showed a K-*ras* gene activation.

The *p53* gene analysis allowed us to detect mutations in 18 of 35 ductal cancers (51.4%). On the contrary, none of various kinds of nonductal exocrine tumors and only one of 12 nonfunctioning endocrine tumors showed a *p53* mutation. Negative findings also were reported by Yoshimoto *et al.* (36) for pancreatic endocrine tumors (mostly benign) and extrapancreatic endocrine tumors. The mutation frequency we found in ductal cancers (51.4%) is slightly higher than the 43.7% figure reported by Scarpa *et al.* (16) using the single-strand conformation polymorphism technique.

Abnormal levels of the *p53* gene product have been found by immunohistochemical methods in 60% of ductal pancreatic tumors (12, 16), and the molecular analysis of samples with immunodetectable protein confirmed in most cases the presence of point mutations within the gene. The mutation frequency we found is comparable to the rate of p53 overexpression observed immunohistochemically. On the other hand, no p53 protein overexpression has been detected in acinar cell carcinomas (17).

All of the *p53* changes we observed were point mutations, with the only exception being a 3-base pair deletion detected in a giant cell carcinoma. Among point mutations only 2 cases produced a stop codon.

All of the *p53* genetic changes were in the highly conserved domains of the protein or at conserved amino acid residues. Ten of 20 mutations were clustered in exon 8, and 5 of 10 were in codon 273 which is a known mutation hot spot in various human tumors.

Of 19 *p53* changes, 11 were base transitions. While transversions were more heterogeneous, 9 of 11 transitions were of the same G:C→A:T type. A prevalence of G:C→A:T substitutions in pancreatic tumors was also observed by other authors (12, 14, 16). On the whole, the mutation spectrum we found in ductal pancreatic cancer (Table 2) appears more complex compared to other tumor types (20, 21). The action of different carcinogens as well as the occurrence of spontaneous base substitutions may contribute to this mutation pattern. In fact, 8 of 9 G:C→A:T transitions occurred at CpG mutation hot spots (1 at codon 248, 5 at codon 273, and 2 at codon 282). Spontaneously occurring deamination at methylated CpG sites is a general mechanism accounting for C→T and G→A changes (37) and has been indicated as an endogenous cause of somatic mutations in *p53* gene (38). However, only 2 of 8 transitions were at CpG sites (codon 175) in the study by Scarpa *et al.* (16), who also failed to detect preferential involvement of specific sites.

The presence of mutations in both K-*ras* and *p53* genes found in 13 ductal cancers (Table 3) suggests a cooperative effect of K-*ras* and *p53* alterations in ductal pancreatic tumorigenesis. K-*ras* activation may represent an early and critical event in such a process (see above). On the other hand, *p53* changes seem to be especially involved in

Table 3 *Gene mutations and histological grading in ductal tumors*

Presence of mutations	G1	G2	G3	Total
None	4	1	0	5
K-*ras* only	5	7	0	12
p53 only	2	1	2	5
K-*ras* + *p53*	2	6	5	13

more severe tumor cell transformation. In fact, all G3 tumors showed *p53* mutations (Table 3), suggesting that these may be relevant to malignancy progression to the higher G3 grade. Finally, our work shows a striking difference at the molecular level between ductal cancers and the remaining pancreatic tumors. In fact, both K-*ras* and *p53* mutations were frequent in ductal cancers (71.4 and 51.4%, respectively), whereas no alterations (with the exception of a single case showing a mutated *p53*) were present in a variety of rare non-ductal tumors we examined. All of the latter tumors involve cell types, either exocrine or endocrine, normally forming pancreatic lobuli, whereas ordinary ductal cancers are essentially made up of cells mimicking mucin-producing cells of extralobular ducts (24). Therefore, the above findings suggest a strong specificity of gene mutations with respect to the different epithelial cell types and compartments of the pancreas. Indeed, specific gene mutations of ductal cells might explain why, among the manifold population of epithelial cells forming the pancreas, a single type, normally accounting for a restricted portion (<10%) of the gland, generates a vast majority (approximately 80%) of its tumors. It may be of interest to investigate whether a similar specificity operates in the tumorigenesis of other organs and tissues with multiple epithelial components.

REFERENCES

1. Boyle, P., Hsieh, C. C., Maisonneuve, P., La Vecchia, C., Macfarlane, G. J., Walker, A. M., and Trichopoulos, D. Epidemiology of pancreas cancer (1988). Int. J. Pancreatol., *5:* 327–346, 1989.
2. Poston, G. J., Gillespie, J., and Guillou, P. J. Biology of pancreatic cancer. Gut, *32:* 800–812, 1991.
3. Korc, M., Meltzer, P., and Trent, J. Enhanced expression of epidermal growth factor receptor correlates with alterations of chromosome 7 in human pancreatic cancer. Proc. Natl. Acad. Sci. USA, *83:* 5141–5144, 1986.
4. Smith, J. J., Derynck, R., and Korc, M. Production of transforming-growth factor α in human pancreatic cancer cells: evidence for superagonist autocrine cycle. Proc. Natl. Acad. Sci. USA, *84:* 7567–7570, 1987.
5. Lemoine, N. R., Lobresco, M., Leung, H., Barton, C., Hughes, C. M., Prigent, S. A., Gullick, W. J., and Kloppel, G. The *erbB*-3 gene in human pancreatic cancer. J. Pathol., *168:* 269–273, 1992.
6. Schmiegel, W., Roeder, C., Schmielau, J., Rodeck, U., and Kalthoff, H. Tumor necrosis factor α induces the expression of transforming growth factor α and the epidermal growth factor receptor in human pancreatic cancer cells. Proc. Natl. Acad. Sci. USA, *90:* 863–867, 1993.
7. 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.
8. 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.
9. Hall, P. A., Hughes, C. M., Staddon, S. L., Richman, P. I., Gullick, W. J., and Lemoine, N. R. The *c-erbB-2* proto-oncogene in human pancreatic cancer. J. Pathol., *161:* 195–200, 1990.
10. Shibata, D., Almoguera, C., Forrester, K., Dunitz, J., Martin, S. E., Cosgrove, M. M., Perucho, M., and Arnheim, N. Detection of c-K-*ras* mutations in fine needle aspirates from human pancreatic adenocarcinomas. Cancer Res., *50:* 1279–1283, 1990.
11. Stork, P., Loda, M., Bosari, S., Wiley, B., Poppenhusen, K., and Wolfe, H. Detection of K-*ras* mutations in pancreatic and hepatic neoplasms by non-isotopic mismatched polymerase chain reaction. Oncogene, *6:* 857–862, 1991.
12. 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 tumour suppressor gene in human pancreatic cancer. Br. J. Cancer, *64:* 1076–1082, 1991.
13. Hohne, M. W., Halatsch, M. E., Kahl, G. F., and Weinel, R. J. Frequent loss of expression of the potential tumor suppressor gene DCC in ductal pancreatic adenocarcinoma. Cancer Res., *52:* 2616–2619, 1992.
14. 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.
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., Iacono, C., and Hirohashi S. Pancreatic adenocarcinomas frequently show p53 gene mutations. Am. J. Pathol., *142:* 1534–1543, 1993.
17. Hoorens, A., Lemoine, N. R., McLellan, E., Morohoshi, T., Kamisawa, T., Heltz, P. U., Stamm, B., Ruschoff, J., Wiedenmann, B., and Kloppel, G. Pancreatic acinar cell carcinoma. An analysis of cell lineage markers, p53 expression, and K-*ras* mutation. Am. J. Pathol., *143:* 685–698, 1993.
18. Lemoine, N. R., Jain, S., Hughes, C. M., Staddon, S. L., Maillet, B., Hall, P. A., and Kloppel, G. Ki-*ras* oncogene activation in preinvasive pancreatic cancer. Gastroenterology, *102:* 230–236, 1992.
19. Bartek, J., Iggo, R., Gannon, J., and Lane, D. P. Genetic and immunochemical analysis of mutant *p53* in human breast cancer cell lines. Oncogene, *5:* 893–899, 1990.
20. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.
21. Caron de Fromentel, C., and Soussi, T. TP53 tumor suppressor gene: a model for investigating human mutagenesis. Genes Chromosomes Cancer, *4:* 1–15, 1992.
22. Cubilla, A. L., and Fizgerald, P. J. Atlas of Tumor Pathology, Series 2, No. 19. Washington, DC: Armed Forces Institute of Pathology, 1984.
23. Kloppel, G. Pancreatic Non-endocrine tumors. *In:* G. Kloppel and P. U. Heitz (eds.), Pancreatic Pathology, pp. 79–113. Edinburgh, Scotland: Churchill Livingstone, 1984.
24. Sessa, F., Bonato, M., Frigerio, B., Capella, C., Solcia, E., Prat, M., Bara, J., and Samloff, M. I. Ductal cancers of the pancreas frequently express markers of gastrointestinal epithelial cells. Gastroenterology, *98:* 1655–1665, 1990.
25. Solcia, E., Sessa, F., Rindi, G., Villani, L., Riva, C., Buffa, R., and Capella, C. Classification and histogenesis of gastroenteropancreatic endocrine tumors. Eur. J. Clin. Invest., *20:* 72–81, 1991.
26. Pellegata, N. S., Losekoot, M., Fodde, R., Pugliese, V., Saccomanno, S., Renault, B., Bernini, L. F., and Ranzani G. N. Detection of K-*ras* mutations by denaturing gradient gel electrophoresis (DGGE): a study on pancreatic cancer. Anticancer Res., *12:* 1731–1736, 1992.
27. Renault, B., van den Broek, M., Fodde, R., Wijnen, J., Pellegata, N. S., Amadori, D., Meera Khan, P., and Ranzani, G. N. Base transitions are the most frequent genetic changes at P53 in gastric cancer. Cancer Res., *53:* 2614–2617, 1993.
28. Sheffield, V. C., Cox, D. R., Lerman, L. S., and Myers, R. M. Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single base changes. Proc. Natl. Acad. Sci. USA, *86:* 232–236, 1989.
29. Myers, R. M., Maniatis, T., and Lerman, L. S. Detection and localization of single base changes by denaturing gradient gel electrophoresis. Methods Enzymol., *155:* 501–517, 1987.
30. Sanger, F., Nicklen, S., and Coulson, A. R. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA, *74:* 5463–5467, 1977.
31. Carbone, D., Chiba, I., and Mitsudomi, T. Polymorphism at codon 213 within the *p53* gene. Oncogene, *6:* 1691–1692, 1991.
32. Grunewald, K., Lyons, J., Frohlich, A., Feichtinger, H., Weger, R. A., Schwab, G., Janssen, J. W. G., and Bartram, C. R. High frequency of Ki-*ras* codon 12 mutations in pancreatic adenocarcinomas. Int. J. Cancer, *43:* 1037–1041, 1989.
33. Tada, M., Omata, M., and Ohto, M. *Ras* gene mutations in intraductal papillary neoplasms of the pancreas. Cancer (Phila.), *67:* 634–637, 1991.
34. Sessa, F., Pellegata, N. S., Bonato, M., Capella, C., Solcia, E., Kloppel, G., and Ranzani, G. N. Mutations of P53 and K-*ras* in intraductal papillary tumors are related to grade of epithelial dysplasia. Digestion, *54:* 308–309, 1993.
35. 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.
36. Yoshimoto, K., Iwahana, H., Fukuda, A., Sano, T., Saito, S., and Itakura, M. Role of *p53* mutations in endocrine tumorigenesis: mutation detection by polymerase chain reaction-single strand conformation polymorphism. Cancer Res., *52:* 5061–5064, 1992.
37. Coulondre, C., Miller, J. H., Farabaugh, P. J., and Gilbert, W. Molecular basis of base substitution hot spots in *Escherichia coli*. Nature (Lond.), *274:* 775–780, 1978.
38. Rideout, W. M., III, Coetzee, G. A., Olumi, A. F., and Jones, P. A. 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes. Science (Washington DC), *249:* 1288–1290, 1990.