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# Neoplasia of the Ampulla of Vater

## *Ki*-ras *and p53 Mutations*

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***Eleven tumors of the ampulla of Vater (5 stage IV and 2 stage II adenocarcinomas, 1 stage II papillary carcinoma, 1 neuroendocrine carcinoma, and 2 adenomas, one with foci of carcinoma) were examined for Ki-ras and p53 gene mutations by single-strand conformation polymorphism analysis and direct sequencing of polymerase chain reaction–amplified DNA fragments. Ki-ras mutations were found in one adenocarcinoma and in the adenoma with foci of carcinoma, both involving mainly the intraduodenal bile duct component of the ampulla. Seven cases showed p53 gene mutations: four advanced-stage adenocarcinomas, the papillary carcinoma, the neuroendocrine carcinoma, and the adenoma with foci of carcinoma. Nuclear accumulation of p53 protein was immunohistochemically detected in the morphologically high-grade areas of the five cancers harboring a p53 gene missense point mutation. The adenomas, the two frame shift–mutated cancers, and the adenomatous and low-grade cancer areas of mutated carcinomas were immunohistochemically negative. Our data suggest that in ampullary neoplasia 1) p53 mutations are common abnormalities associated with the transformation of adenomas and low-grade cancers into morphologically high-grade carcinomas, and 2) Ki-ras mutations are relatively less frequent and might be restricted to tumors originating from the bile duct component of the ampulla. (Am J Pathol 1993, 142:1163–1172)***

Ampullary epithelial neoplasias include benign (5%) and malignant tumors (95%) centered in the region of the papilla of Vater.[1,2] They represent 5% of all gastrointestinal tumors but account for up to 36% of the surgically operable pancreatoduodenal tumors.[2] Originally defined on topographic grounds, the term ampullary neoplasia also carries a histogenetic significance; it implies origin from the epithelium of one of the three anatomical components of the ampulla of Vater, either the ampulla (common channel) or the intraduodenal portions of the bile duct or of the pancreatic duct. It is of clinical importance to differentiate ampullary cancers from those arising from periampullary structures such as pancreas, duodenum, and extraduodenal bile duct, because in a large number of cases, their prognosis seems to be significantly better.[2–4] Assumption of the existence of an adenoma-carcinoma sequence in ampullary cancer is based almost entirely on the morphological observations of adenomatous areas in a variable proportion (55 to 91%) of malignant tumors and of the presence of foci of carcinoma within adenomas.[1,5–7]

There is increasing evidence that the accumulation of molecular abnormalities activating the oncogenic potential of protooncogenes and inactivating the growth repressor function of tumor suppressor genes underlies the multistage processes of tumorigenesis and progression of malignancy.[8] These phenomena have been particularly well characterized in colorectal cancer.[9–12] *ras* family protooncogenes and p53

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This work is dedicated by the Italian authors to the memory of their friend and teacher Dr. Pietro Novelli of the Pathology Department of Verona University.

tumor suppressor gene are among the best-studied cellular genes whose alterations have a role in the pathogenesis of human cancer.

The *ras* family includes three active members, Ha-*ras*, Ki-*ras*, and N-*ras*.[13] The 21-kd *ras* proteins have been suggested to belong to the group of G proteins, which are involved in the process of signal transduction across the cell membrane. They are, in fact, located on the inner surface of the plasma membrane and possess guanine nucleotide binding and hydrolytic activities both *in vitro* and *in vivo*.[13] Activating mutations at codons 12, 13, or 61 have been detected in a variety of different human neoplasms with variable frequencies.[14] Preferential involvement of Ki-*ras* has been demonstrated in up to 50% of colon and lung adenocarcinomas, while activated N-*ras* genes predominate in hematopoietic neoplasms.[14,15] By far the highest incidence of the Ki-*ras* mutations, almost all involving the first or second base of codon 12, has recently been observed in pancreatic carcinomas[16] and in distal bile duct carcinomas.[17,18]

Mutations of the p53 gene,[19] located on chromosome 17p13,[20] are the most common genetic abnormalities in human malignancies.[21] This gene encodes a 53-kd nuclear phosphoprotein with a short half-life that negatively regulates cell growth and proliferation.[22] The recessive tumor suppressor role[23] of this gene has been suggested both at the experimental level[24–26] and by the demonstration in several human tumors of the monoallelic loss of variable portions of the short arm of chromosome 17,[27,28] including the p53 locus, together with its consistent association with the mutation of the remaining p53 allele.[21,27–30] The mutated proteins show a prolonged half-life, and their accumulation in the nuclei of affected cells is immunohistochemically detectable using p53 specific monoclonal antibodies.[30–33]

The aim of this study was to explore the presence and pattern of mutations of Ki-*ras* and p53 genes in 11 cases of ampullary neoplasia. We used a simple and sensitive method for detection of structural alterations of DNA including point mutations, which is single-strand conformation polymorphism (SSCP) analysis of DNA fragments obtained by polymerase chain reaction (PCR) (PCR-SSCP analysis[34,35]). This method has been successfully used for the detection of DNA polymorphisms in Alu repeats,[35] of gene mutations in different genetic diseases (cystic fibrosis, neurofibromatosis type 1, hemophilia B and B1 variant of Tay-Sachs disease),[35] as well as of somatic mutations in the *ras* family genes[36] and in the p53 genes of various cancers.[37–41]

We demonstrated that 1) p53 gene mutations and allelic losses are commonly found in ampullary carcinomas and are associated with morphologically high-grade and advanced stage cancers, 2) Ki-*ras* mutations are relatively less frequent and are associated with tumors that have prevalent involvement of the intraduodenal bile duct component of the ampulla.

## Materials and Methods

### Pathological Samples and Controls

Of the 135 patients having periampullary tumors who underwent surgery at the Verona University, Italy, during the period 1971 to 1991, 88 were diagnosed as having epithelial tumors of the ampulla of Vater. Eleven of these cases, for which frozen material was available, are the object of the present study (Table 1). They included 7 intestinal-type adenocarcinomas, 1 papillary carcinoma, 1 neuroendocrine carcinoma, and 2 villous adenomas. One of the adenomas (case 9) contained foci of low- to high-grade adenocarcinoma. The high-grade carcinoma in 6 also showed low-grade areas characterized by glands lined by cells with abundant pale cytoplasm. The neuroendocrine carcinoma had been described in a previous report.[42] All tumors protruded into the duodenal lumen and involved all three anatomical components of the ampulla (common channel, intraduodenal pancreatic and bile ducts). Tumors 4, 9, and 10, however, showed a preferential involvement of the bile duct component. Five cancers were ulcerated: three adenocarcinomas of intestinal type (cases 3, 5, and 6), the adenocarcinoma with prominent areas of squamous differentiation (case 4), and the neuroendocrine carcinoma (case 8). Of the four nonulcerated adenocarcinomas, three were of intestinal type (cases 1, 2, and 7), and one (case 10) was of papillary type. The two adenomas were nonulcerated. All samples were obtained at the time of pancreatoduodenectomy. Formalin-fixed paraffin-embedded tissues were used for conventional histological and immunohistochemical studies. A portion of the tumors and, in eight cases, samples of normal duodenal or pancreatic tissue from the same patients, were snap-frozen in liquid nitrogen for DNA analysis. A frozen sample of lymph node metastasis was also available for case 8. Before DNA extraction,[43] 5-μ cryostat sections were stained with hematoxylin and eosin to histologically check the material for the DNA studies. In all samples neoplastic cells accounted for more than 70%, except for cases 3 and 6, in which the cancer component was approximately 50% and 10 to 20%, respectively. Additional

**Table 1.** *Ampullary Tumors: Clinico-Pathological Data*

| Case | Age/ Sex | Macroscopy | Diagnosis | Local Extension | Stage* | p53 Mutation | Follow-Up (Months) |
|---|---|---|---|---|---|---|---|
| AT1 | 70/F | Nonulcerated, 3.5 cm | Adenocarcinoma M/D | Duodenal serosa, pancreas | IV/N+M | + | AD/7 |
| AT2 | 59/M | Nonulcerated, 1.5 cm | Adenocarcinoma P/D† | Microinfiltration of duodenal submucosa | II/N– | – | AW/15 |
| AT3 | 73/M | Ulcerated, 2 cm | Adenocarcinoma M/D | Duodenal serosa, pancreas | IV/N+ | + | AW/13 |
| AT4 | 66/M | Ulcerated, 1.5 cm | Adenocarcinoma P/D†‡ with squamous areas | Duodenal serosa, pancreas | IV/N– | – | DOD/6 |
| AT5 | 67/M | Ulcerated, 2.5 cm | Adenocarcinoma P/D† | Periduodenal fat, pancreas | IV/N+ | + | DOD/4 |
| AT6 | 68/F | Ulcerated, 2.5 cm | Adenocarcinoma M/D† | Duodenal serosa, pancreas | IV/N– | + | DOD/16 |
| AT7 | 46/F | Nonulcerated, 1 cm | Adenocarcinoma M/D | Microinfiltration of duodenal submucosa | II/N– | – | AW/37 |
| AT8 | 66/M | Ulcerated, 2 cm | Neuroendocrine carcinoma | Duodenal serosa, pancreas | IV/N+M | + | DOD/6 |
| AT9 | 53/M | Nonulcerated, 2 cm | Villous adenoma‡ with malignant foci | Intraductal | I/N– | –/+§ | AW/17 |
| AT10 | 66/M | Nonulcerated, 2 cm | Carcinoma M/D‡ papillary type | Duodenal submucosa | II/N– | + | AW/3 |
| AT11 | 60/F | Nonulcerated, 2 cm | Villous adenoma, severe dysplasia | Intraductal | I/N– | – | AW/22 |

Abbreviations: M/D, P/D: moderately and poorly differentiated; AD, alive with disease; AW, alive and well; DOD, died of disease.
* Staging according to Yamaguchi et al (ref. 2). Case 1 had liver metastasis, case 8, had liver and brain metastasis.
† Areas of adenoma were present.
‡ Tumors 4, 9, and 10 involved preferentially the common bile duct. In case 4, the terminal third of the common bile duct was involved.
§ The p53 positivity was confined to the high-grade carcinomatous area.

cryostatic sections were also obtained for immunohistochemical analysis.

## *PCR-SSCP Analysis for Detection of Ki-*ras *and p53 Gene Mutations*

PCR-amplified fragments corresponding to exons 5 to 8 of the p53 gene and to exons 1 and 2 of the Ki-*ras* gene were analyzed by the SSCP method.[34,35] The positive cases were further analyzed by sequencing PCR products. To check the sensitivity of the SSCP analysis, all cases were also independently subjected to PCR sequencing of Ki-*ras* exons 1 and 2, starting from genomic DNA.

### *Oligonucleotide Primers*

The oligonucleotides were synthesized by the phosphoramidite method using a DNA synthesizer model 380A (Applied Biosystem Co., Tokyo, Japan) and purified through oligonucleotide purifying cartridge columns following the manufacturer's instructions. The sequences of primers used to PCR-amplify Ki-*ras* exons were (5′–3′): exon 1, GGCCTGCTGAAAATGACTGA and GTCCTGCACCAGTAATATGC; exon 2, TTCCTACAGGAAGCAAGTAG and CACAAAGAAAGCCCTCCCCA. The sequences of primers, derived from published sequences,[44] used to PCR-amplify p53 exons were (5′–3′): exon 5, TTCCTCTTCCTGCAGTACTCC and GCCCCAGCTGCTCACCATCG; exon 6, CGATGGTGAGCAGCTGGGGC and AGTTGCAAACCAGACCTCA; exon 7, TCCTAGGTTGGCTCTGAC and CAAGTGGCTCCTGACCTGGA; exon 8, CCTATCCTGAGTAGTGGTAA and CCTGCTTGCTTACCTCGCT.

### *PCR*

The primers were 5′-end-labeled with [$\gamma$-$^{32}$P]ATP as previously described.[45] The PCR mixture contained 0.25 pmol each of the labeled primers, 2 nmol each of the four deoxynucleotides, 0.1 μg of genomic sample DNA, and 0.25 U of Taq polymerase in 10 μl of the buffer specified in the Gene Amp kit (Perkin-Elmer Cetus, Emeryville, CA). Thirty cycles of the reaction at 94 C, 55 C, and 72 C, respectively, for 0.5, 0.5, and 1 minute were run in a Thermocycler (Perkin-Elmer Cetus).

### *SSCP*

A portion of the PCR mixture (1 μl) was withdrawn, mixed with 9 μl of 0.1% $NaDodSO_4$ and 10 mmol/L EDTA, and analyzed on a 5% polyacrylamide gel to check the product of the amplification reaction. Then 2 μl of this solution were mixed with 2 μl of 95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol, heated at 80 C, and applied (1 μl/lane) to a 6% polyacrylam-

ide gel (20 × 40 × 0.03 cm, 0.5 cm/lane) containing 90 mmol/L Tris-borate, pH 8.3, 4 mmol/L EDTA. To obtain the maximum sensitivity of the SSCP analysis, the samples were also run on two additional 6% polyacrylamide gels, with the respective addition of 5% and 10% glycerol. The three different types of gels were electrophoresed at 30 W for 2 to 6 hours in two different conditions, the first at room temperature (20 to 25 C) using fans for cooling, the second at 4 C in a cold room. The gels were dried on filter paper and exposed to x-ray film at −80 C for 0.5 to 12 hours with an intensifying screen (DuPont, Dreieich, Germany). Of the different experimental conditions used, both for the Ki-*ras* and the p53 amplified DNA fragments, the best resolution of normal and shifted bands was achieved running the samples in 5% glycerol gels at room temperature.

## *Direct DNA Sequencing*

The DNA samples that showed mobility shifts of single-stranded DNA fragments were subjected to direct sequencing by the asymmetric PCR method.[46] To enrich the mutated sequences, the shifted bands were eluted from the gel, amplified, and then sequenced according to the method described by Suzuki et al.[47] The wild-type bands of the same cases were also sequenced as an internal control. Briefly, the small area of the gel corresponding to the position of the appropriate band was cut out, immersed in 25 μl of distilled water in a 0.5 ml centrifuge tube, and heated at 80 C for 15 minutes. The water extract (5 μl) was subjected to asymmetric amplification by the PCR (50 cycles) in a mixture (50 μl) containing an uneven molar ratio of the two primers (10 to 50:1). Asymmetric PCR products were freed from unincorporated nucleotides and primers using a Centricon 30 microconcentrator (Amicon, Beverly, MA) and then subjected to the sequencing reaction as described.[46] After annealing to a 5′-labeled primer, the sequencing reactions were carried out using the termination mixtures of a Sequenase kit (United States Biochemical Corporation, Cleveland, Ohio) and analyzed on a 6% polyacrylamide gel containing 7 mol/L urea. The sequence abnormalities were confirmed by opposite-strand sequencing.

## *Immunohistochemical Analysis*

All cases were characterized both in frozen and in paraffin sections for the presence of p53 protein using the anti-p53 monoclonal antibody PAb1801 (Oncogene Science Inc., Manhasset, NY), that recognizes a denaturation-resistant epitope between amino acids 32 and 79.[48] The frozen sections were incubated for 1 hour in a humid chamber with the anti-p53 antibody, diluted 1:50 in phosphate-buffered saline containing 20% rabbit serum and 0.1% saponin. The same procedure was used for the paraffin sections, except for the first antibody incubation, which was performed overnight. Antibody localization was determined using standard avidin-biotin-complex–peroxidase method on formalin-fixed sections, and alkaline phosphatase anti-alkaline phosphatase technique on cryostatic sections.

# *Results*

The clinico-pathological characteristics of the patients and the results of the genetic study are summarized in Tables 1 and 2.

## *Mutations of Ki-*ras *Gene*

Two of the three cases which mainly involved the intraduodenal bile duct component of the ampulla,

**Table 2.** *Ampullary Tumors: Mutations of Ki-*ras *and p53 Genes*

| | *ras* mutations (exons 1 and 2) | | | p53 mutations (exons 5–6–7–8) | | | |
|---|---|---|---|---|---|---|---|
| Case | Codon | Sequence mutation | Protein mutation | Exon | Codon | Sequence mutation | Protein mutation |
| AT1 | | | | 7 | 245 | GGC → GAC | Gly → Asp |
| AT2 | | | | | | | |
| AT3 | | | | 6 | 220 | TAT → TGT | Tyr → Cys |
| AT4 | 12 | GGT → GAT | Gly → Asp | | | | |
| AT5 | | | | 7 | 245 | GGC → CGC | Gly → Arg |
| AT6 | | | | 5 | 179 | CAT → TAT | His → Tyr |
| AT7 | | | | | | | |
| AT8 | | | | 6 | 219 | CCC → del C | Stop 246* |
| AT9 | 12 | GGT → GCT | Gly → Ala | 5 | 173 | GTG → ATG | Val → Met |
| AT10 | | | | 6 | 209 | AGA → delAG | Stop 214* |
| AT11 | | | | | | | |

* The deletion resulted in a frame shift error and the creation of a non-sense stop codon in the position indicated.

cases 4 and 9, showed an abnormally migrating band at the SSCP analysis of the Ki-ras exon 1 (Figure 1). In both cases the mutations involved codon 12 (Figure 2), whose sequence alteration impairs the intrinsic guanosine triphosphatase activity of the p21-*ras* protein.[13,17] All remaining cases also scored negative at the PCR-direct sequencing for the Ki-*ras* exon 1. All cases were negative both at SSCP and PCR sequencing analyses of the Ki-*ras* exon 2.

In our experimental conditions, the SSCP analysis for the Ki-*ras* gene was sensitive and specific, as evaluated by the 100% concordance between the results obtained by direct sequencing *versus* SSCP analysis for both exons 1 and 2 (2 positive and 10 negative cases for exon 1 and all negative for exon 2). Moreover, we demonstrated that SSCP is able to recognize all six possible mutations of the first and second base of codon 12 (Figures 1 and 3), which represent over 90% of the Ki-*ras* mutations found in periampullary (pancreatic and terminal bile duct) cancers.[16–18]

## *Mutations of p53 Gene*

A shifted band at the SSCP analysis of the p53 gene (Figure 4) was present in 7 cases: four advanced adenocarcinomas (cases 1, 3, 5, 6), the early-stage papillary cancer (case 10), the adenoma with areas of carcinomatous transformation (case 9), and the neuroendocrine carcinoma (case 8). The lymph node metastasis of the latter case showed a shifted band in exon 6 at the same level as the one observed in the primary tumor (Figure 4). No p53 mutations at SSCP analysis were found in the advanced carcinoma with squamous differentiation (case 4), in 2 carcinomas limited to the duodenal submucosa (cases 2 and 7), and in 1 adenoma (case 11). In 5 cases the p53 mutations were represented by single nucleotide changes resulting in

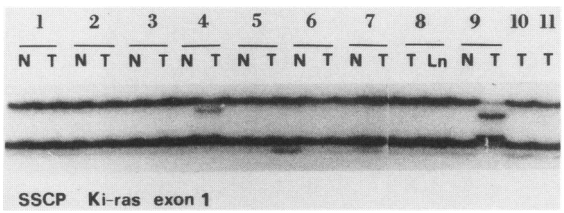


**Figure 1.** *SSCP analysis of PCR-amplified Ki-*ras *exon 1 from normal (N) and tumor (T) tissues. Ln, lymph node metastasis. Shifted bands are present in cases 4 and 9. The germline bands in tumor 9 are clearly under-represented, reflecting the loss of one Ki-*ras *allele. The samples were run on 6% acrylamide gels with the addition of 5% glycerol at room temperature. Case numbers correspond to those shown in the tables.*

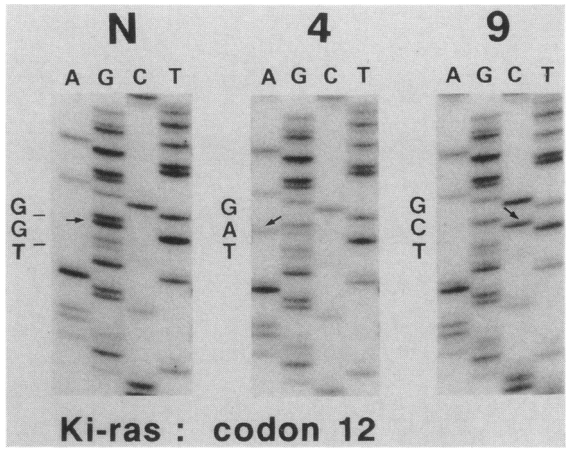


**Figure 2.** *Antisense sequences of the mutated bands of cases 4 and 9, eluted from the gel shown in* **Figure 1.** *N is the sequence of the SSCP upper normal band of case 4, eluted from the same gel. In both cases the mutation involves the second base of codon 12.*

missense mutations. In the remaining 2 cases, respectively, a 1- and 2-bp deletion (Figure 5) were the genetic defects. Four mutations were at G:C and one at A:T bp. The transitions were G to A in two instances, one A to G, and one C to T. The last point mutation was a G to C transversion. None of the mutations were at a CpG site. In cases 1, 5, 8, and 10, the wild-type bands were clearly underrepresented at SSCP analysis (Figure 4), reflecting the loss of the normal allele.[47] However, in cases 3

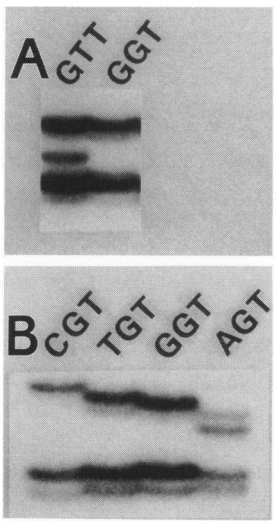


**Figure 3.** *SSCP analysis of PCR-amplified Ki-*ras *exon 1 performed on 6% acrylamide gels with the addition of 5% glycerol at room temperature. GGT is the wild-type codon 12 sequence and identifies the lanes containing DNAs from normal tissues. The lane marked GTT contains PCR-amplified DNA from a pancreatic cancer carrying a G to T mutation at the second base of codon 12* **(A).** *The lanes marked CGT, TGT, and AGT contain PCR-amplified DNAs from three different cancer cell lines (PSN1, Lu65, and A549) (see ref. 36) carrying the corresponding first-base mutations of codon 12* **(B).** *Note that different sequence abnormalities correspond to different shifts.*

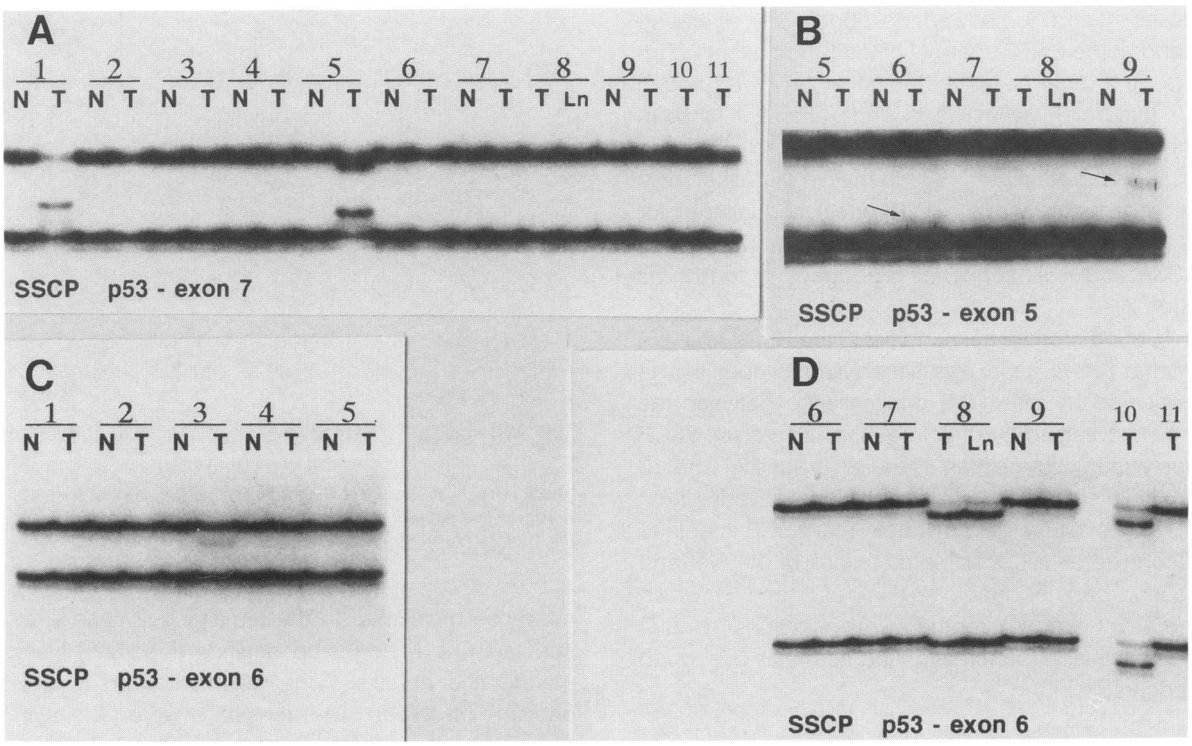


**Figure 4.** *SSCP analysis of PCR-amplified exons of p53 gene from normal (N) and tumor (T) tissues. Ln, lymph node metastasis. The germline bands in tumors 1, 5, 8, and 10 are clearly under-represented, reflecting the loss of one p53 allele. A 6% acrylamide gel was used for exon-6 amplified samples 1 to 5; all the other gels were added of 5% glycerol. Case numbers correspond to those shown in the tables.*

and 6, the loss of the normal p53 allele could not be excluded, because the sample contained a significant number of normal cells. The somatic nature of the mutations was demonstrated by their absence in normal tissues of the same patients.

## *Comparison of Macroscopic Aspect, Histological Type, and Extension of Tumors with p53 Mutations*

In 5 cases of ulcerated carcinoma, 3 adenocarcinomas and the neuroendocrine carcinoma presented a p53 mutation and were ≥2 cm. The p53-negative adenocarcinoma (case 4) showed areas of squamous differentiation and measured 1.5 cm. All the ulcerated cancers were extended to the duodenal serosa and pancreas (stage IV). In 4 cases of nonulcerated carcinoma, 2 had a p53 mutation: case 1 was an adenocarcinoma, measuring 3.5 cm, extended to the duodenal serosa and pancreas (stage IV), and case 10 was a papillary cancer, measuring 2 cm, limited to the duodenal submucosa (stage II). The p53-negative adenocarcinomas, cases 2 and 7, measured less than 2 cm and were limited to the duodenal submucosa (stage II). The 2 adenomas were both nonulcerated and measured 2 cm. Case 11 was a pure adenoma and scored negative for p53 and Ki-*ras* mutations. Case 9 was an adenoma with foci of carcinoma and showed both a Ki-*ras* and a p53 mutation. However, the intensity of the p53-mutated band was much lower than the Ki-*ras* mutated band (Figures 1 and 4), suggesting that only a minor part of the Ki-*ras*-mutated neoplastic population also possessed the p53 mutation.

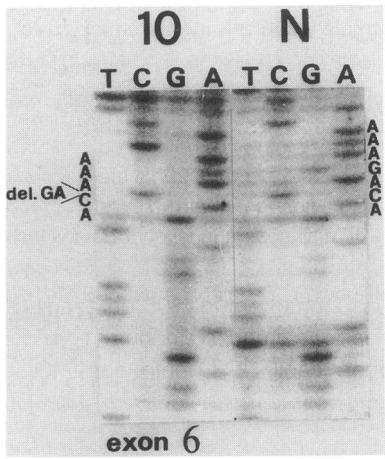


**Figure 5.** *Sequence of the shifted band of case 10, eluted from the gel shown in* **Figure 4.** *N is the sequence of the SSCP upper normal band of the same case. A 2-bp deletion is the genetic defect.*

### *Nuclear Accumulation of p53 Protein*

The results of immunohistochemical study were identical for frozen and for paraffin sections except in the case of the adenoma with foci of carcinoma (case 9), in which no cancer foci were present in the paraffin block. The cancer cells of the five cases harboring a p53 gene missense point mutation showed nuclear stain with pAb1801 monoclonal antibody. The two cases in which a frame shift mutation was the p53 genetic defect (cases 8 and 10) and all of the p53 mutation-negative cases displayed no stain at all. In all cancers with an adenomatous component, as well as in the adenoma with foci of carcinoma (case 9), the immunohistochemical positivity was confined to the carcinomatous areas, whereas the adenomatous areas were homogeneously negative (Figure 6). The low-grade carcinomatous areas of cases 6 and 9 were also immunohistochemically negative.

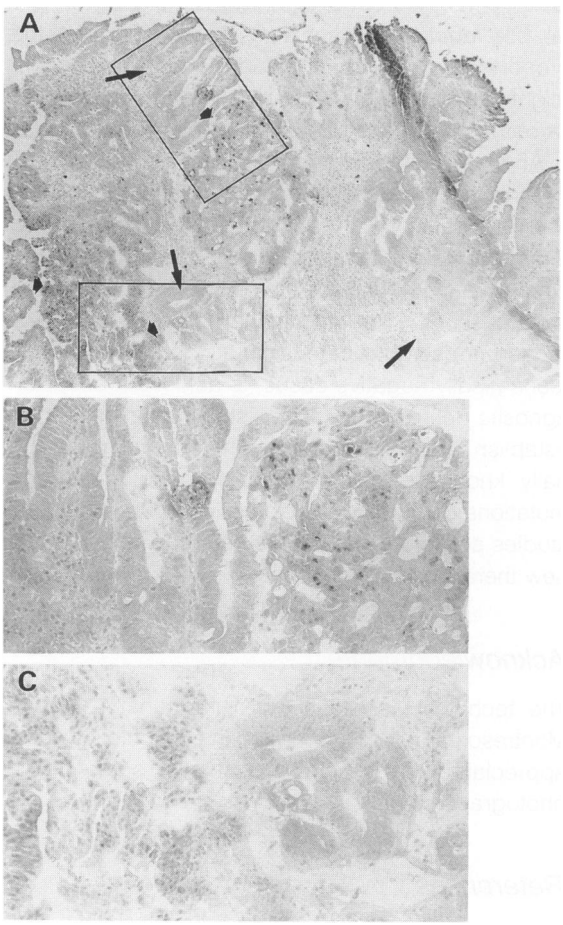


**Figure 6.** *Case 9.* **A:** *Immunohistochemical stain with monoclonal antibody pAb1801 of a frozen section of the tissue utilized for genetic analysis. High-grade cancer cells (arrowheads) show p53-immunohistochemical nuclear stain, whereas the adenoma/low grade carcinoma cells are negative (arrows).* **B** *and* **C:** *Enlargement of the squared areas in* **(A)**.

## *Discussion*

The most important findings of our study can be summarized as follows. 1) p53 mutations are common abnormalities in ampullary cancer and are associated with the transformation of adenomas and low-grade cancers into morphologically high-grade carcinomas. 2) p53 mutations are associated with immunohistochemically detectable nuclear accumulation of the p53 protein, with the exception of cases in which frame shift mutations deeply alter the gene sequence. 3) Ki-*ras* mutations are relatively less frequent and the two tumors harboring such abnormality were characterized by the prevalent involvement of the bile duct component of the ampulla.

The question arises whether our results may underestimate the frequency of gene mutations, for the SSCP method may not detect every possible mutation in a given DNA fragment. This is not the case for the Ki-*ras* gene sequences. In fact, it has been demonstrated that SSCP is able to recognize all possible mutations in the first and second base of codon 12 (this paper) as well as many mutations in exons 1 and 2 of the *ras* family genes, including codons 13, 61, and 18.[36] Moreover, the screening independently conducted with SSCP and PCR sequencing in our cases for both the Ki-*ras* exons 1 and 2 had results that were perfectly concordant. On the other hand, our results may slightly underestimate the frequency of p53 gene alterations, whereas the mutations found in cancers are widespread along the gene. However, SSCP has been used successfully to detect an extremely wide range of p53 mutations in cancer tissues and cell lines both in our laboratories (89 different sequence abnormalities)[38–41] and in others.[37] Another possible cause of underestimation of p53 mutations lies in the fact that our analysis was restricted to the p53 sites most often implicated in human tumors.[21] Evidence from studies on solid tumors suggest that other regions could be mutated, although at a very low frequency.[49] Overall, we believe that, even though the possibility of an underestimation of the p53 mutation frequency must be recognized, it is not significant.

Mutations in the p53 gene clustered in more advanced cases (5 of 6 cases) in terms of both local growth and extension of the tumor. In fact, most of the cases with p53 mutations were ≥2 cm in diame-

ter and extended to the duodenal serosa and pancreas. Of note was the fact that the only case with advanced disease not harboring a p53 gene mutation showed prominent areas of squamous differentiation. The fact that the p53 gene/protein abnormality was only detected in moderate- and high-grade carcinomatous areas of the adenoma with malignant foci suggests that such a mutation might have a role in the progression of a precancerous, nonmalignant lesion into a highly malignant one. The observation of the p53 immunohistochemical negativity of the adenomatous and low-grade cancerous areas in positively staining carcinomas further supports this hypothesis. A similar scenario has been proposed for the adenoma-carcinoma neoplastic progression in colon.[30,33]

All of the mutations involved evolutionary highly conserved domains of the p53 protein, as with those reported in colonic and other malignancies.[21] Different from p53 mutations in colon cancer, which mainly occur at CpG dinucleotides[21] (67%), in none of our cases did the mutation involve these sites. This observation might have an important pathogenetic implication. CpG sites, in fact, are preferential targets for point mutations in mammalian cells, as spontaneous errors during DNA replication, presumably due to the deamination of methylated cytosine residues.[50,51] Mutations occurring outside of these sites could be related to specific endogenous or exogenous carcinogens. Indeed, liver cancers of subjects exposed to food containing aflatoxin $B_1$ showed p53 mutations similar to those caused by this substance in mutagenesis experiments.[52,53] Notably, ampullary cancer occurs with a statistically unexpected frequency in patients with particular occupational exposures to as yet unidentified chemical carcinogens.[54]

The recessive tumor suppressor role of the p53 gene in ampullary cancer is suggested by the monoallelic loss of the p53 gene locus in 4 cases and its association with the mutation of the remaining p53 allele. In the cancers displaying both a normal and a mutated p53 allele, this finding most probably reflects hemizygosity for the mutated allele, the normal one being present only in contaminating normal cells. However, true p53 heterozygosity of the tumor cells, with the mutated allele acting as a dominant negative oncogene,[55] cannot be formally ruled out.

Among the different factors relevant for prognosis, it has been suggested that the histogenesis of ampullary cancers influences survival.[56] Attempts have been made to recognize the epithelium of origin using the mucin staining pattern of tumors, as compared with the different mucin production of the different components of normal ampulla.[56] The site of origin is usually determined by the location of the main bulk of the tumor. It is often a very difficult task and can be resolved in about 40% of the cases, in which the tumor is small and confined to one of the three ductal components of the ampulla. Both in our experience and in that of Yamaguchi et al,[2] most of the cases presumably originated from the common channel, and only a minor proportion originated from the intraduodenal portion of the pancreatic or the bile duct. It is intriguing that the proportion of Ki-*ras* mutated ampullary cancers is about 15 to 20% of the cases, as has also been observed by others.[18,57] In our cases, the mutations were found in two of the three cases in which the main tumor was confined to the bile duct structures. These cases were intestinal-type adenocarcinomas, whereas the negative one showed a papillary pattern of growth. These data, together with the observation of a high frequency of Ki-*ras* mutations in distal bile duct malignancies,[17,18] suggest that the proportion of Ki-*ras* mutated ampullary cancers might correspond to that originating from the epithelia of the intraduodenal portion of bile duct.

An important result from our study is the demonstration that monoclonal antibody pAb1801 detects a large majority of the p53-mutated carcinomas. Simple and low-cost immunohistochemistry is sufficient to detect the presence of p53 abnormalities in most cases. On the other hand, the PCR-SSCP approach is useful to detect mutations in immunohistochemically negative cases, to achieve a better diagnostic definition in positive-staining cases, and to establish the nature of post-therapy relapses. Finally, knowledge of the specific spectrum of gene mutations could help in addressing epidemiological studies seeking etiological factors and might reveal new therapeutic approaches.

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