

## K-Ras and Dpc4 Mutations in Chronic Pancreatitis: Case Series

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**Aim** To assess whether alterations in the *K-ras*, *p53*, and *DPC4* genes are present in pancreatitis, a potential precancerous condition that can progress to pancreatic adenocarcinoma. To investigate the alterations occurring at hot spots of *K-ras* (exon 1), *p53* (exons 5 and 7), and *DPC4* (exons 8, 10 and 11).

**Methods** In 10 patients with acute and 22 with chronic pancreatitis, without pancreatic intraepithelial neoplasia (PanIN), DNA was isolated from paraffin embedded tissue samples. The extracted DNA was analyzed by polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis, single-strand conformation polymorphism (SSCP) analysis, and DNA sequencing.

**Results** In acute pancreatitis samples no mutations were found in any of the investigated genes. In 7 out of 22 samples of chronic pancreatitis nucleotide substitution at exon 1 of *K-ras* (five at codon 12 and two at codon 13) were found. No mutations in *p53* (exons 5 and 7) were detected. Two samples had nucleotide substitutions at exons 8 and 11 of *DPC4*, introducing STOP signal and change in the amino acid sequence, respectively. One chronic pancreatitis sample displayed simultaneous mutations in *K-ras* (exon 1, codon 12) and *DPC4* (exon 8, codon 358).

**Conclusion** Mutations of *K-ras* and *Dpc4* genes can accumulate already in non-malignant, inflammatory pancreatic tissue, suggesting its applicability in monitoring of further destruction of pancreatic tissue and progression into malignancy.

Pancreatitis is an inflammatory disease of the pancreas, characterized by the progressive tissue destruction, leading both to exocrine and endocrine insufficiency. Acute pancreatitis can manifest as a benign condition with minimal abdominal pain and hyperamylasemia, or can have a fulminate course due to the development of infective pancreatic necrosis and multisystem organ failure (1).

On the other hand, chronic pancreatitis is characterized by the progressive destruction of pancreatic parenchyma and its replacement by variable amounts of fibrous tissue (2). A characteristic histological profile displays a fibrosis in association with the foci of inflammatory cells, the regions of acinar cell degradation, and the areas of ductal cell proliferation (3).

Diagnosing pancreatitis at an early stage is a clinical challenge, because the frequency of pancreatic ductal adenocarcinoma in patients with pre-existing chronic pancreatitis is significantly higher than in the general population. It has been shown that chronic pancreatitis increases the risk of developing pancreatic cancer 10 to 20 times (4). Progression model of pancreatic ductal adenocarcinoma at the molecular level shows that it always harbors *K-ras*, *p53*, and *p16* alterations, and also mutations in the *DPC4* (deleted in pancreatic cancer gene, locus 4) (5-7). In contrast to pancreatic cancer, the molecular mechanism responsible for acute and chronic pancreatitis is poorly understood. Some improvement has been made with the discovery of the genetic nature of hereditary pancreatitis (8,9). The studies of molecular alterations occurring in acute and chronic pancreatitis have mainly included the investigation of alterations in cationic trypsinogen gene, *K-ras*, *p53*, or cystic fibrosis transmembrane conductance regulator gene (*CFTR*) (8,10-14).

Since pancreatitis represents a precancerous state in a considerable number of cases, we assumed that the alterations in more than one gene could be associated with the genetic basis

of the disease and the genes involved in the development of pancreatic adenocarcinoma could be mutated already in pancreatitis. Therefore, in collected tissue samples of acute and chronic pancreatitis, we investigated the alterations occurring at hot spots of three different genes (*K-ras*, *p53*, *DPC4*) which are members of different signaling pathways.

## Material and methods

### Patients and genomic DNA preparation

Postmortem pancreatitis samples were obtained from 10 patients with acute pancreatitis and 22 patients with chronic pancreatitis between 1998 and 2003. For the purposes of this study, the samples were retrieved from the archival tissue files of the Zagreb University Hospital Center, Department of Pathology. Histological sections cut from 10% buffered formalin-fixed paraffin-embedded blocks were routinely stained with hematoxylin and eosin. Diagnosis of acute and chronic pancreatitis without pancreatic intraepithelial neoplasia (PanIN) was confirmed by the same pathologist (J. J. R.) for all samples.

Paraffin-embedded sections (4  $\mu\text{m}$ ) were fixed to the slides, and 2-3 sections were used for one-step DNA extraction by TaKaRa DEXPAT TM (TaKaRa Bio Europe, Inc, Gennevilliers, France) according to manufacturer's instructions. Three separate DNA extracts were prepared from each paraffin embedded block.

### Polymerase chain reactions

Genomic DNA from the sections was amplified by polymerase chain reactions (PCR) in a final reaction volume of 25  $\mu\text{L}$  containing 1x HotMaster Taq Buffer (Eppendorf, Hamburg, Germany), 50  $\mu\text{mol/L}$  of each dNTP (Eppendorf), 5 pmol of forward and reverse primers, 1 U HotMaster Taq DNA polymerase (Eppendorf), and 2.5  $\mu\text{L}$  of extracted DNA (50-100 ng).

PCR was performed under conditions of an initial denaturation at 94°C for 2 minutes, 30-40 cycles consisted of denaturation (30 seconds at 94°C), annealing (30 seconds at 51-58°C, depending on the primer) and elongation step (30 seconds at 72°C), and a final extension step at 72°C for 7 minutes. Reactions were performed in a BioSystems Thermocycler 2700 (Applied BioSystems, Foster City, CA, USA). Each PCR batch was tested three times and always included a blank to which no DNA had been added, to ensure that no contamination of samples had occurred. Sequences of primers designed for PCR reaction are listed in Table 1. The PCR products were visualized in ethidium bromide-stained 3% agarose gel.

All pancreatitis samples were screened for mutation within coding region by two analyses: PCR-restriction fragment length polymorphism analysis (RFLP) for detection of previously documented mutation based on restriction site, and PCR-single strand conformation polymorphism analysis (SSCP) for searching for other mutations in the analyzed samples.

#### PCR-RFLP

PCR-RFLP analysis was used in order to detect mutations in the hot spots of *K-ras* (codons 12 and 13), *p53* (codons 175 and 248), and *DPC4* (codons 358, 412, and 493) genes.

Two PCR amplification rounds were performed for mutational analysis at exon 1 of the *K-ras* gene (15). PCR products were digested with *Bst* NI (New England BioLabs, Inc., Frank-

furt, Germany) for testing mutation at codon 12, and with *Bgl* I (BioLabs) for revealing mutation at codon 13, under the reaction conditions recommended by the supplier.

*p53* mutations were identified using PCR amplification, followed by restriction with *Cfo* (BioLabs) enzyme (exon 5, codon 175), and with *Msp*I (BioLabs) enzyme (exon 7, codon 248).

The screen for mutation in the *DPC4* gene involved exons 8 (codon 358), 10 (codon 412), and 11 (codon 493), under the same conditions as published in our previous study (16).

RFLP products of analyzed genes were separated on Spreadex EL 300 or EL 400 gels (Elchrom Scientific, Cham, Switzerland). A 6 µL of restriction mixture, together with 1 µL of loading buffer (Eppendorf) were loaded onto gel, which was subsequently stained with Sybr Gold (Molecular Probes, Leiden, Netherlands) for 30 minutes, destained in distilled water, and analyzed on UV transilluminator at 254 nm.

#### PCR-SSCP

All specimens of pancreatitis tissue were screened for other mutations within the coding region of *K-ras* (exon 1), *p53* (exons 5 and 7), and *DPC4* (exons 8, 10 and 11) by PCR-SSCP analysis.

A 5 µL of PCR product was mixed with 7 µL of formamide with 10 mmol/L NaOH. The mixture was heated at 95°C for 5 minutes and then immediately placed on ice. Samples were separated by electrophoresis in 8% polyacrylamide gels at +4°C. The gels were silver stained.

**Table 1.** Mutational analysis of three genes in 22 chronic pancreatitis tissue samples

Gene	Codon	Primer sequences 5'→3'	No. of mutated samples	Type of mutations
<i>K-ras</i>	12	ACTGAATATAAACTTGTGGTAGTTGGACCT, TCAAAGAATGGTCCTGGACC, TAAATATGTCGACTAAAACAAGATTTACCTC	5	GGT>GAT, TGT, GTT, GCT or GAT
<i>K-ras</i>	13	ACTGAATATAAACTTGTGGTAGTTGGCCCTGGT, ACTCATGAAAATGGTCAGAGAACCCTTTAT, TCAAAGAATGGTCCTGCACC	2	GGC>GAC, TGC or GAC
<i>p53</i>	175	TGTTCACTTGTGCCCTGACT, CAGCCCTGTCGTCTCTCC	0	-
<i>p53</i>	248	CCTCATCTTGGCCCTGT, CCAGGGGTCAGCGCAAGCA	0	-
<i>DPC4</i>	358	CTCCTGAGTATTGGTGTTC, CTTGCTCTCTCAATGGCTTC	1*	c.1203G>T
<i>DPC4</i>	412	TTGCGTCAGTGTATCGACAA, GATAGCTGGAGCTATTCCAC	0	-
<i>DPC4</i>	539	TCTGTCAGCTGCTGCTGGAA, GGTTGTGGGTCGCAATCGG	1	c.1744T>G

\*Sample also positive to *K-ras*/12 mutation.

**Sequence analysis of PCR products**

PCR product of the sample which revealed aberrant PCR-SSCP pattern was directly re-amplified from the Spreadex EL 1200 gel and then purified using a QIAquick column (QIAGEN Companies, Valencia, CA, USA) according to the manufacturer's instructions. One microgram of purified PCR product in volume of 50 µL was sequenced by ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction FS Kit and an ABI PRISM 377 DNA sequencer (Applied Biosystems).

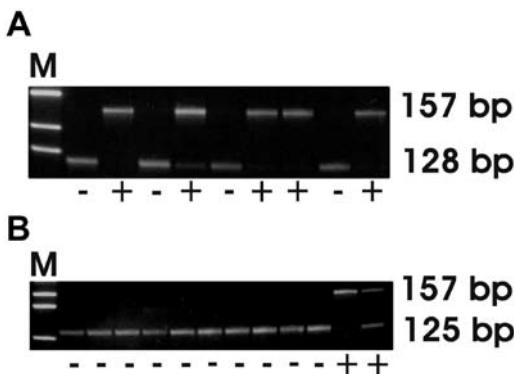
**Results**

***K-ras***

PCR-RFLP analysis of exon 1 of the *K-ras* showed nucleotide substitution at codon 12 in 5 samples (Figure 1A) and at codon 13 in 2 samples of chronic pancreatitis (Figure 1B). One sample with mutation at codon 12 of *K-ras* was also positive to *DPC4* mutation (Table 1). None of the samples had patterns showing aberrant SSCP variants.

***p53***

PCR-RFLP analysis of the *p53* gene revealed no mutations at exon 5 (codon 175) and exon 7 (co-

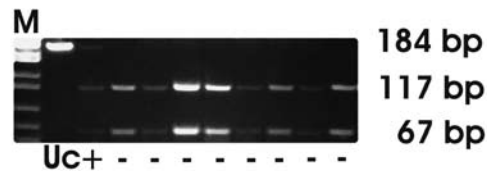


**Figure 1.** *K-ras* gene profile (exon 1) of chronic pancreatitis samples after RFLP analysis. (A) 157 bp fragment represents mutation (+) at codon 12 and 128 bp fragment represents wild type allele (-). (B) 157 bp fragment represents mutation (+) at codon 13 and 125 bp fragment represents wild type (-) allele. M – molecular weight DNA marker IX (Boehringer, Mannheim, Germany).

don 248) in all 32 pancreatitis samples (Table 1). Moreover, no aberrant profile in PCR-SSCP analysis of these samples was detected.

***DPC4***

A single chronic pancreatitis sample showed the nucleotide substitution GGA→TGA, introducing STOP signal instead of Gly at exon 8 (Figure 2). The same sample also had a mutation at codon 12 of the *K-ras* gene (Table 1). Additional PCR-SSCP analysis of *DPC4* indicated the presence of a mutation in another sample of chronic pancreatitis (Figure 3A). Sequence analysis confirmed that indication, revealing GTA→GGA substitution at exon 11 (codon 539) which causes a change in the amino acid sequence (Val→Gly) (Figure 3B).

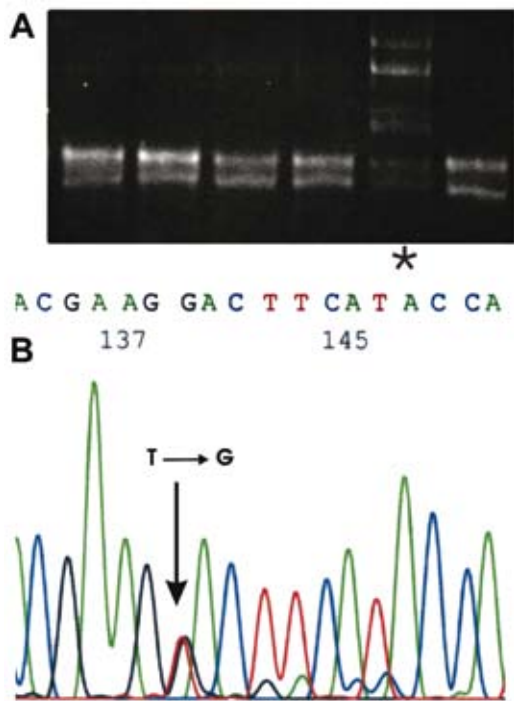


**Figure 2.** *DPC4* gene profile (exon 8) of chronic pancreatitis samples after RFLP analysis. Mutant allele (+) lacks *Mnl* I restriction site at codon 358, resulting with 184 bp fragment and wild type allele (-) is cut by *Mnl* I at codon 358 resulting with 117 bp and 67 bp fragments. M – molecular weight DNA marker M3 (Elchrom Scientific); uc – uncut PCR product.

**Discussion**

This study presents the evidence of alterations in *K-ras* and *DPC4* genes, but not in *p53* gene, in paraffin embedded tissue samples of chronic pancreatitis. Inflammation within the pancreatic ducts could cause alterations in numerous genes that belong to different pathways (17). We speculate that the mutations we observed could be involved in malignant transformation and therefore mark pre-malignant pancreatic state.

The analysis performed on acute pancreatitis samples showed the absence of mutations at any analyzed locus of all tested genes. This finding is consistent with the fact that most cases of acute pancreatitis recover without long-term se-



**Figure 3.** *DPC4* gene mutation (exon 11) in one chronic pancreatitis sample. (A), PCR-SSCP analysis of the DNA fragment (asterisk indicates aberrant SSCP pattern). (B), Sequence analysis of the same DNA fragment points out single nucleotide substitution at codon 539.

quel, with only a minority of cases progressing to chronic pancreatitis (1,18,19). The time to accumulate mutations due to inflammation is thus shortened, and cells are destroyed rather than altered.

Of all genes tested in this study, *K-ras* exhibited the highest frequency of mutations in chronic pancreatitis samples; 5 samples (23%) were positive in the codon 12 and 2 (9%) in the codon 13. These results were not unexpected because the presence of *K-ras* mutation is not rare in patients with chronic pancreatitis, as validated by several groups, which found *K-ras* mutations in 39% (20), 28% (21), or 34% (22) of patients with chronic pancreatitis. These data indicate that *K-ras* alterations could be taken as a risk factor for pancreatic ductal adenocarcinoma, although the sequence of events and the time required for conversion to an established malignancy is very hard to define. In contrast to our results, two groups came to the conclusion that this mutation is ex-

ceedingly uncommon within the paraffin-embedded pancreatitis specimens. They demonstrated the total absence of *K-ras* (codons 12 and 13) mutations in samples of chronic pancreatitis (2,12).

Furthermore, we also analyzed the *p53* gene at mutational hot spots, exon 5 and 7. No *p53* gene mutations were discovered in the analyzed pancreatitis samples, which is consistent with prior observations that these mutations occur in chronic pancreatitis tissues only occasionally (11,23), in contrast to pancreatic carcinomas, where the occurrence is almost 50% (23-25). On the other hand, our results are in opposition to those obtained by one research group (12) presenting the evidence for early occurrence of *p53* (exons 5-9) mutations in chronic pancreatitis. Eight of 80 samples displayed alterations in *p53* detected by PCR-SSCP analysis and confirmed by DNA sequence analysis. The authors suggested that mutations in *p53* could also occur at an early stage of pancreatic disease, without morphological evidence of pancreatic cancer, as has been found in benign or precancerous lesions in other human tissues, such as hyperplastic prostatic epithelium (26) and fibroadenoma of the breast (27). The accumulated data suggest that we should continue to investigate some additional exons of *p53* in a larger series of patients with chronic pancreatitis.

In this study, we also focused on the *DPC4* gene, whose protein mediates signals from the family of ligands of transforming growth factor- $\beta$  signaling pathway (28). In 2 of 22 examined samples, mutations in C-terminal region of *DPC4* were found. One sample revealed nucleotide substitution at codon 358 (exon 8) which is incorporated into the mutation cluster region of the gene (29), and another sample had a single nucleotide substitution at codon 539 (exon 11). This is an interesting finding because previous molecular studies showed that inactivating mutation of the *DPC4* gene is a late genetic change in the multistep progression model of pancreatic ductal

adenocarcinomas contributing to tumor progression (30-33). Mutations occurring at the exons of *DPC4* gene have never been found in pancreatitis tissues. One chronic pancreatitis sample in our study displayed simultaneous mutations in *K-ras* (codon 12) and *DPC4* (exon 8) genes. To our knowledge, only one group detected coinciding alterations of *K-ras* and *DPC4* genes in non-tumoral pancreatic disease (34). But in that study, pancreatic juice of patients with chronic pancreatitis was sampled and only codon 12 of *K-ras* was analyzed.

Our study demonstrated that *DPC4* mutations might be present already in non-malignant tissue such as that of chronic pancreatitis, which is considered to have a potential for malignant transformation. The significance of these mutations and their consequences should be clarified in the future. Nevertheless, the finding of mutated *DPC4* in chronic pancreatitis suggests an involvement of mutation in the progression of the disease toward malignancy.

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