Detection of K-*ras* Point Mutations in the Pancreas by Constant Denaturing Gel Electrophoresis Using the DCode™ System

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Introduction

Genetic alterations/variations are the cause underlying many nonneoplastic and probably all neoplastic diseases. Today the number of reports associating genetic alterations with cancer (Williams et al. 1996) and other diseases is rapidly increasing. In many cases the exchange of only one or a few bases, so-called point mutations, give rise to severe disease.

We are specifically interested in ductal adenocarcinoma of the exocrine pancreas, which is the second most frequent cause of death by digestive tract cancer (Parker et al. 1996). Nearly all of these tumors bear a point mutation in codon 12 of the K-*ras* protooncogene (Chu 1997, Klöppel 1994). We wanted to determine the frequency of these mutations in ductal lesions and associated tissues of tumor-free pancreases. We used microdissection followed by a sensitive single-step PCR regime to amplify the exon 1 of the K-*ras* gene. Point mutations were detected by constant denaturing gel electrophoresis (CDGE) (Børresen et al. 1991) using the DCode universal mutation detection system.

Methods

Archived, formalin-fixed, paraffin-embedded pancreas tissue samples of patients without pancreatic ductal adenocarcinoma were drawn from the files of the Institute of Pathology. Evaluation of conservation was done by histological routine procedures. A section was stained with hematoxylin and eosin (H & E) and used as reference. Cells were microdissected from an immediate corresponding unstained 10 µm thick section using a micromanipulator (Narishige). For the aspiration of the lesions or morphologically normal cells, capillaries of 10–25 µm diameter were used. The section was overlaid with TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Aspiration only of the buffer served as contamination control. The aspirated cells/buffer were transferred to 5 µl proteinase K digestion buffer (20 µg proteinase K/ml in 10 mM Tris/HCl, 0.5% Nonidet P40) in PCR reaction vials. Digestion was performed for 10 min at 55°C to demask the DNA followed by an inactivation of the proteinase K (15 minutes at 96°C). To amplify the K-*ras* DNA, PCR buffer was added to a final concentration of 12 mM Tris/HCl, pH 8.0, 50 mM KCl, 0.1% Nonidet, and 0.2 mM each dNTP and 2 mM $MgCl_2$. The reactions contained, in a total volume of 25 µl, 50 ng of 3'-primer (CTA TTG TTG GAT CAT ATT CG), 100 ng of 5'-primer (CGCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCC CGCCCC CTG AAT ATA AAC TTG TGG), and 0.5 U of *Taq* DNA polymerase (Boehringer Mannheim). PCR was performed using a program of 45 cycles (45 sec at 95°C, 40 sec at 58°C, 30 sec at 70°C). The final extension was 5 min at 70°C. PCR products were analyzed via a constant denaturing gel electrophoresis (CDGE) using the DCode universal mutation detection system (Bio-Rad Laboratories, Inc.). Gels contained 35% denaturant, 1x TAE, and 7.5% polyacrylamide (30:1), were run for 240 min at 200 V, 60°C, and were silver stained. For sequencing, the deviant bands were cut out of the gel ,and the DNA was eluted by soaking in TE. After reamplification and TA cloning (Invitrogen Corporation), several clones were sequenced (ABI PRISM dye terminator cycle sequencing kit/ ABI PRISM 310 automated sequencer, Applied Biosystems).

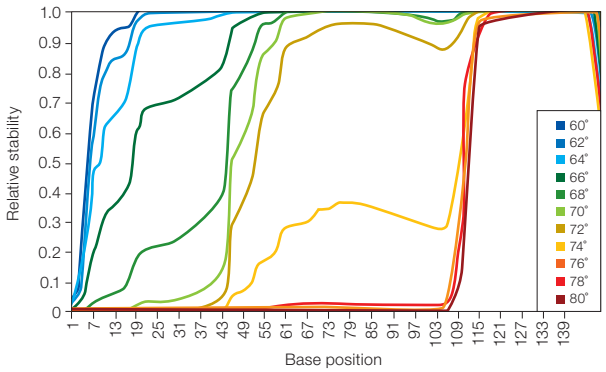

Fig. 1. POLAND plot of the K-*ras* exon1 PCR product wild-type (wt) sequence (codon 12=GGT). The relative stability of an open base pair is plotted as a function of position in sequence and temperature (°C, see inset). The K-*ras* PCR fragment shows 3 melting domains. From position 1–50 is the lowest melting domain, from position 50–105 is the intermediate melting domain (containing codon 12), and the highest melting domain is created by a GC clamp from position 105–142.

Results

The calculated melting profile of the generated PCR fragment shows that it contains three melting domains. The putative mutations were in the second melting domain, which could not be analyzed without a GC clamp (the most stable domain) (Figure 1). The difference in melting profiles of the mutant and wt K-*ras* PCR-products should allow separation via CDGE (Figure 2). As controls, we generated mutated sequences

by PCR and cloning. Figure 3 shows a 20–50% denaturant perpendicular DGGE gel run to determine the optimal denaturant concentration for the CDGE analysis, which was determined to be 35% (Figure 3). A 35% CDGE gel was run with samples from all mutations of codon 12 leading to an amino acid exchange and wt PCR products (Figure 4).

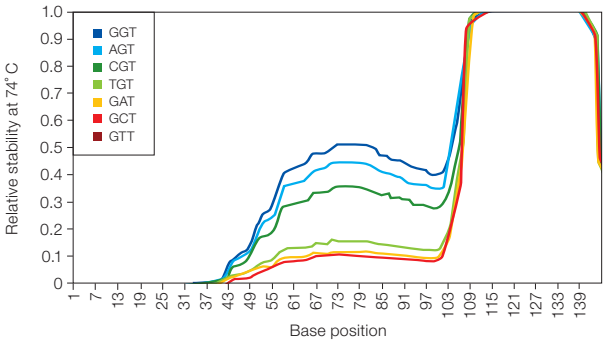

Fig. 2. Stability of *K-ras* codon 12 sequence variants for 74°C showing the best differences of the relevant codon 12 sequences. It predicts that G>A or G>T mutations in position 1 or 2 of the codon will reduce the stability of the second melting domain, which will lead to slower migration than wt. It is predicted that GAT and TGT cannot be separated from each other.

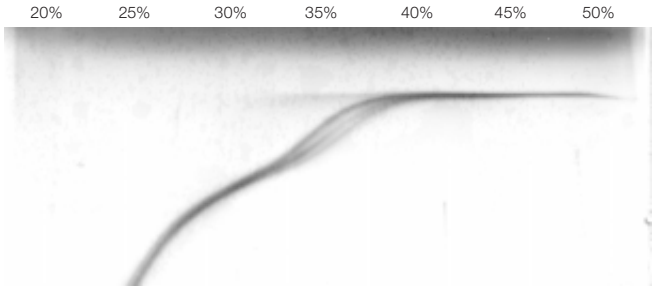

Fig. 3. Perpendicular gradient (20–50%) DGGE gel showing the optimal denaturant concentration for CDGE analysis, determined to be 35%.

We analyzed 1,069 samples of 71 pancreases. Mutations were detected in 16 samples of 12 pancreases. With the exception of 2 samples of normal duct cells (243 screened) all the mutation-positive samples were from lesions (605 screened). All acinar cells screened (221) were negative. Figure 5 shows a typical result with one of the positive samples. In contrast to this low frequency, we could detect K-*ras* point mutations in more than 95% of ductal adenocarcinoma of the pancreas (data not shown).

Discussion

Mutations of K-*ras* were thought to be quite rare in pancreases free of ductal adenocarcinoma. However, we detected mutations in 16 samples from normal pancreases. This finding

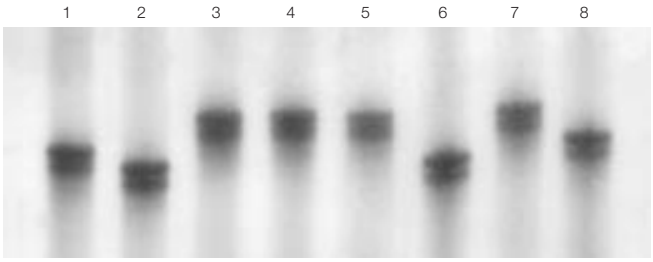

Fig. 4. 35% CDGE gel. Mutations of codon 12 leading to an amino acid exchange were run in parallel with wt PCR products. Lanes 1 and 8, wt control; lanes 2–7, mutant samples.

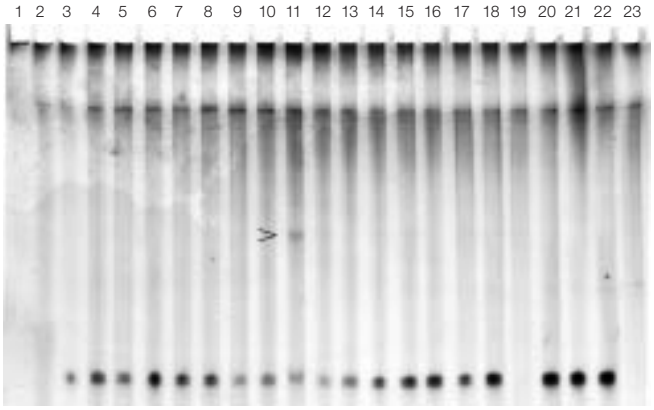

Fig. 5. 35% CDGE gel. Lanes 1, 2, 19 and 23, contamination control (aspirated buffer; lanes 3–17, mutant samples; lanes 20–22, wt control. >, heteroduplex band caused by mutated K-*ras*.

raises the question of which role K-*ras* mutations play in the series of genetic alterations leading to ductal adenocarcinoma of the pancreas. We believe it is a very early genetic change promoting cancer but not causing it. As K-*ras* mutations can be found in morphologically normal cells and in tumor-free pancreases, caution should be used when interpreting findings of mutations in pancreatic juice or feces.

References

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