# Detection of K-ras mutations in pancreatic cancer samples collected by fine needle aspiration biopsy: an intermediate report on the first results and experience

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Dedicated to Professor Přemysl Frič, MD, DSc, on the Occasion of his 75th Birthday

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***Abstract.*** *Background and aims. Somatic mutations in K-ras oncogene are widely considered as early events in pancreatic cancerogenesis, with frequencies of close to 90 % in developed pancreatic cancers. Their occurrence in premalignant intraepithelial stages suggests potential for diagnostic and screening purposes of risk individuals by means of diagnostic testing based on fine needle aspiration biopsy. Recently, several groups have demonstrated detection of K-ras mutations in pancreatic resection tissue. This paper presents a report on sensitive detection of K-ras mutations directly in biopsy tissue from pancreatic cancer patients.*

*Methods. Fine needle aspiration biopsy samples were taken from a total of 35 individuals diagnosed with primary suspicious pancreatic mass during endoscopic ultrasonography examination. The aspirates were evaluated cytologically and were subjected to molecular-genetic analysis of K-ras mutations. The definite malignant status of the tumours was subsequently confirmed from resections following a surgery and/or a long-term follow-up.*

*Results. Of 30 patients with confirmed pancreatic cancer status, 21 (70 %) exhibited malignant positivity for both, cytology and K-ras mutation test, 2 patients (6.7 %) had negative cytology tests while carrying a K-ras mutation and 3 patients (10 %) had positive cytology with no K-ras mutations. In 4 patients (13.3 %) with subsequently confirmed pancreatic cancer, neither of the two tests were positive. Sensitivity of single cytologic evaluation was 80 %, sensitivity of cytology combined with K-ras mutation test reached 90 %. As a result of cases with positive cytology and no K-ras mutations, the original technique of parallel examination of dissected bioptic tissue by the two tests was modified to a tumour-targeting approach, in which K-ras mutations are detected directly from cytology smears.*

*Conclusion. The results obtained from this initial study confirmed a high frequency of K-ras mutations in biop-*

*tic tissue samples from pancreatic tumours. Fine needle aspiration biopsy was confirmed as a suitable method of sampling for purposes of molecular diagnostics testing in addition to classic cytology test.*

***Key words:*** *pancreas, pancreatic cancer, fine needle aspiration biopsy, mutation detection, K-ras, early detection.*

*Šálek C, Zavoral M, Benešová-Mináriková L, Jelínková M, Traboulsi E, Hrabal P, Nosek V, Minárik M. Stanovení mutací K-ras ve vzorcích karcinomu pankreatu získaných aspirační biopsií tenkou jehlou: zpráva o prvních zkušenostech a dosavadních výsledcích. Folia Gastroenterol Hepatol 2004; 2 (4): 150 - 155.*

*Souhrn. Somatické mutace v onkogenu K-ras jsou detekovány v časných stadiích pankreatické kancerogeneze; jejich frekvence v pokročilém karcinomu pankreatu dosahuje 90 %. Přítomnost K-ras mutací v premaligních intraepiteliálních lézích skýtá potenciál pro screening rizikových osob. Tato studie prezentuje zkušenosti se senzitivní metodou detekce K-ras mutací přímo z bioptického materiálu získaného tenkojehlovou biopsií pankreatu.*

*Metodika. K endosonografickému vyšetření doplněnému tenkojehlovou biopsií bylo referováno 35 pacientů se suspektním ložiskem pankreatu. Tkáňový materiál byl hodnocen cytologicky a odeslán k molekulárně-genetické analýze mutačního spektra onkogenu K-ras. Konečná diagnóza maligního procesu byla potvrzena z chirurgických resekátů nebo dle dlouhodobého klinického průběhu onemocnění.*

*Výsledky. Z 30 pacientů s potvrzenou diagnózou karcinomu pankreatu 21 (70 %) vykazovalo pozitivitu v cytologickém nálezu i v testu na přítomnost K-ras mutací, 2 pacienti (6,7 %) měli negativní cytologický nález, zatímco byli nosiči mutací onkogenu K-ras, a u 3 pacientů (10 %) vyšlo pozitvní cytologické vyšetření, ale mutace v onkogenu K-ras detekovány nebyly. Čtyři pacienti (13,3 %) s následně potvrzenou diagnózou karcinomu pankreatu zůstali negativní v obou testech. Senzitivita samotného cytologického vyšetření byla 80 %, v kombinaci s evaluací mutačního spektra onkogenu K-ras dosáhla 90 %. Pro nález pozitivních cytologických závěrů u pacientů bez detekovaných K-ras mutací byla původní metodologie, kdy bioptický materiál byl vyšetřován paralelně cytologicky a molekulárně-geneticky, změněna a K-ras mutace jsou nyní detekovány přímo v materiálu z cytologických nátěrů.*

*Závěr. Studie potvrdila vysokou incidenci K-ras mutací v tkáních karcinomu pankreatu. Tenkojehlová biopsie byla potvrzena jako vhodná metoda k získávání tkáňového materiálu pro cytologické i molekulárně-genetické vyšetření. Význam molekulárně-genetické analýzy onkogenu K-ras jasně potvrdily dva případy, kdy přes negativitu cytologického nálezu se maligní proces demonstroval přítomností K-ras mutací. Kombinace cytologických a molekulárně-genetických metod zvyšuje diagnostickou senzitivitu, jejíž další elevaci a vliv na včasnou detekci raných - ještě kurabilních - stadií lze očekávat po zahrnutí molekulárních markerů v genech p16, DPC a p53.*

***Klíčová slova:*** *Pankreas, karcinom pankreatu, tenkojehlová aspirační biopsie, detekce mutací, K-ras, včasná detekce.*

Despite significant progress in research and a generally better understanding of the molecular and genetic basis of malignant diseases acquired over the past decade, pancreatic cancer remains a mostly fatal disease (14). Late diagnosis of advanced stages of pancreatic cancer is the major cause of its extremely negative prognosis. The most critical issue for a surgical resection, which represents the only method of treatment, is uncovering the very early stages of pancreatic cancer or better yet, detecting premalignant lesions referred to as PanIN - pancreatic intraepithelial neoplasia (8). Most currently applied diagnostic approaches rely on evaluation of morphological changes in pancreatic tissue in combination with histology/cytology examination of samples obtained by fine-needle aspiration (FNA) (5). The development of pancreatic cancer follows a distinct path from normal ductal epithelia, inflammatory tissue, PanIN phase I - III up to the carcinoma (7). This path is accompanied by sequential accumulation of genetic changes (mostly point mutations, gene amplifications and allelic deletions). In order to increase diagnostic sensitivity of the FNA cytology, several papers have demonstrated detection of selected genetic changes in DNA material obtained from pancreatic duct brushings, percutaneous biopsies, or plasma as potential molecular markers for PC (4,11,18,19,23).

An early event in pancreatic carcinogenesis is activation of K-ras oncogene by somatic point substitution (1). This alteration can already be detected already in PanIN-1A lesions as well as in chronic pancreatitis and therefore represents an independent risk fac-

tor for pancreatic cancer. In advanced pancreatic cancer, K-ras mutation is found in nearly to 90 % of cases, therefore considered as a potential molecular marker for early detection of pancreatic cancer (15). Following the initial K-ras activation, a number of other genetic abnormalities take place. PanIN-1A and PanIN-1B phases are characterized by overexpression of Her-2/neu oncogene, which is found in 50 % of pancreatic neoplasms (5). Increased Her-2/neu expression, however, is a result of higher transcription rate rather than gene amplification, rendering Her-2/neu not a good therapeutic target (6). Aside from the above-mentioned oncogenes, there are a number of tumour suppressor genes affected by genetic alterations during the pancreatic cancer transformation process. Among them, p16 (INK4A) tumour-suppressor is already inactivated in transition from PanIN-1B to PanIN-2 phases (3). The p16 is inactivated in almost 95 % of cases of invasive pancreatic cancer, therefore also represents a suitable molecular marker.

The most important goal is to uncover patients with chronic pancreatitis that are at a higher risk of developing pancreatic cancer. K-ras mutation is detected in about one third of chronic pancreatitis aspirates (2,16), and patients with mutated K-ras oncogene and loss of p16 expression are considered to be carriers of independent risk factors for development of pancreatic cancer (17,21). Other tumour-suppressor genes from the pancreatic cancerogenesis such as p53, DPC4 and BRCA2 become inactivated in a later, PanIN-3, phase with overall lower frequency, hence, their potential for screening of high-risk individuals is lower (20).

In the current paper, we describe initial results, discuss experimental issues and share some experience acquired with detecting K-ras mutations in samples obtained by FNA.

## Methods

The thirty-five patients with pancreatic mass (21 males, 14 females, aged 39 - 76, median 63) underwent initial examination with endoscopic ultrasonography guided FNA in order to confirm malignant diagnosis or to differentiate between cancer and inflammatory pseudo-tumour status. FNA biopsies were taken from pancreatic mass, mostly located in the head of the pancreas, a minority of lesions originated in the body of the gland. The ultimate diagnosis was determined based on subsequent surgery or a continuing follow-up. The acquired aspirates were divided into two parts where the first part was evaluated by standard cytopathology and the second part was immediately frozen for molecular-genetic analysis.

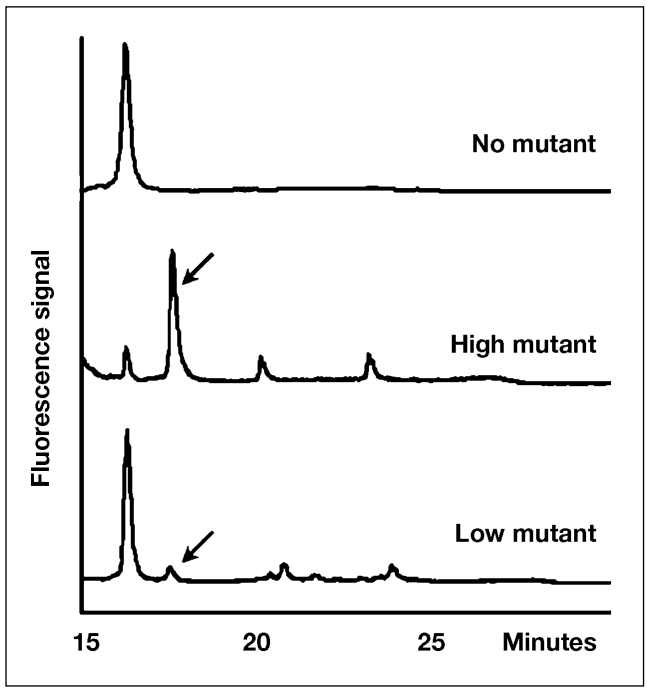


Figure 1
**Illustration of result from mutation detection analysis in case of different fractions of mutated DNA copies in the tissue material. Normal tissue without mutations (A), cancerous tissue with large fraction of mutated cells (B), most common case with low fraction of mutated cells (C).**

Results from cytopathology were classified into three groups: 1) positive findings where the smears were diagnostic of malignant or benign disease; 2) inconclusive or borderline findings where not enough ductal epithelia was present to state diagnosis, or where epithelial atypia did not allow to distinguish cytologically pancreatic cancer from severe chronic pancreatitis; 3) negative findings where no ductal epithelia was detected.

During molecular-genetic analysis, K-ras hotspot codons 12 and 13 of exon 1 were examined for the presence of mutations. Genomic DNA was isolated from the frozen bioptic tissue using JetQuick isolation kit (Genomed GmbH, Loehne, Germany). Extracted DNA was subjected to PCR amplification with fluorescently labeled PCR primers using optimized conditions for amplification of K-ras target sequence (see Table 1). Mutant controls were prepared by PCR mutagenesis by amplification of a wildtype DNA with a special extension on one of the PCR primers. The extension included the mutated base in place of the wildtype base. Following PCR amplification, mutant heteroduplexes were formed by heating and reanne-

Table 1
**Optimized conditions for PCR amplification of K-ras sequence and mutation detection by CGCE technique (13).**

| Target | Primer sequence | Theoretical melting temperature | CGCE temperature range |
|---|---|---|---|
| K-ras | 5'-atgactgaatataaacttgtg-3'<br>5'-FL-[GC]-<br>CCTCTATTGTTGGATCATATTC-3' | 70 °C | 52 - 50 °C |
| [GC] denotes a high-melting clamp:<br>CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG<br>FL denotes labelling by fluorescein | | | |

aling and analyzed on a multi-capillary array analyzer (MegaBACE 1000, Amersham Biosciences, Sunnyvale, California, USA) using periodically cycling temperature gradient (12). The genetic analyzer was equipped with a Caddy plate loading robot (Watrex Praha, Czech Republic) for unattended operation. The separation took place in a standard denaturing gel matrix (MegaBACE long-range matrix, Amersham Biosciences) containing 7M urea. The running conditions included injection for 120 seconds at 3 kV and running at 6 kV for 90 minutes. The cycling gradient temperature profiles were created using MBCS software version 2.0 (Genomac International, Praha, Czech Republic). CGCE running conditions used in this work are listed in Table 1.

The ethical committee of the Institute for postgraduate medical education has approved this study and all patients enrolled have signed informed consent to genetic tests performed on their samples.

## Results

Genomic DNA was extracted from 34 out of the 35 originally collected samples. One patient with negative cytology was excluded from the study for high probability of no ductal epithelia in the tissue aspirate. Of the remaining 34 patients a decisive diagnosis of pancreatic adenocarcinoma was confirmed in 30 cases, chronic pancreatitis in 2 cases, 1 case was a benign pancreatic tumour and 1 mesenchymal tumour (malignant fibrous histiocytoma). The results are summarized in Table 2. In the group of 30 patients with confirmed PC status, 21 patients (70 %) showed positivity for both FNA cytology test as well as K-ras mutation test. A positive cytopathology without positive K-ras test was obtained from 3 patients (10 %), while 2 patients (7 %) exhibited presence of K-ras mutation with negative results from cytology. For the remaining 4 pancreatic cancer patients (13 %), neither of the two tests was positive and the malignancy was only confirmed at the surgery. All detected K-ras mutations were located in codon 12 of exon 1. In one patient the original positive K-ras mutation status from the FNA biopsy was later confirmed in an autopsy sample. The same codon 12 mutation was found in both, biopsy as well as autopsy samples.

Table 2
**Summary of results of cytology test with K-ras mutation detection test.**

| | **K-ras positive** | **K-ras negative** | **Total** |
|---|---|---|---|
| Cytology positive | 21 (70 %) | 3 (10 %) | 24 (80 %) |
| Cytology negative | 2 (7 %) | 4 (13 %) | 6 (20 %) |
| Total | 23 (77 %) | 7 (23 %) | 30 (100%) |

Sensitivity of cytology evaluation was 80 %, sensitivity of cytology combined with evaluation of K-ras mutation status increased to 90 %. However, the difference between both methods was not statistically significant for difference of only two patients between the two groups.

## Discussion

The overall frequency of K-ras mutations found in pancreatic cancer aspirates was 76.7 %, confirming the widely acknowledged significant incidence. The frequencies reported in literature were mostly within a range from 75 to 100 %, with frequency between 61 % and 80 % in pancreatic juice (4,15), 70 - 73 % in pancreatic duct brushings (11), and 80 - 100 % in FNA aspirates (22). Compared to those values, FNA frequency found in this work was slightly lower. This was most likely due to suboptimal processing of the samples. The originally applied method of splitting the bioptic tissue in two parts and examining the parts by the two separate methods (cytology vs. molecular-genetics) clearly introduces a possibility of

inconsistence in results in case of sample heterogeneity. Similarly, the absence of K-ras mutations in the 3 cases of positive cytology leads to a need of examining DNA from the same material, which was evaluated by a pathologist. Therefore, in the next phase, DNA will be isolated directly from cellular smears stained by May-Grünwald-Giemsa technique on which cytopathological diagnosis is stated. This technique avoids genotoxic liquids during staining procedure so samples are optimal for subsequent genetic analysis. With such a tumour-targeting approach, we expect to increase the current frequencies of positive K-ras detection, but more importantly, to increase the potential for detecting other prospective molecular markers such as p16 mutations or DPC allelic deletions, which naturally occur with lower frequencies.

In contrast to the tumour-targeting approach, mutation detection sensitivity is the most important factor for applying the molecular marker screening in pancreatic cancer diagnostic screening. The issue of mutations detection sensitivity is the key in finding small populations of mutated cells in an excess of normal cells (10). This is a legitimate concern in bioptic tissue samples, where often only a fraction of mutated cells is captured in a surrounding of normal unaltered tissue or blood cells. An example of the difference in the fraction of mutated cells is shown in Figure 1. The normal tissue with no detection of mutated DNA (Figure 1A) shows a single PCR product for the wildtype K-ras sequence. A sample containing a large portion of mutated cells (clean cancerous tumour) produces a heteroduplex pattern with the most significant peak of the mutated PCR sequence (Figure 1B). The most common scenario is the one with a low fraction of mutated DNA copies. Such a sample also produces a pattern of heteroduplexes with the most significant peak from the wildtype PCR sequence followed by lower intensity mutant PCR sequences (Figure 1C). With a sufficient level of sensitivity the mutation pattern from Figure 1B is clearly distinguishable from the single peak pattern representing normal tissue in Figure 1A. Based on our previous experience, we estimate the current sensitivity limit of our technique (heteroduplex separation in temperature gradients) at 5 - 10 % of mutated DNA copies (cancer cells) in an excess of 95 % of normal DNA copies (normal cells) (13). Although this part of our study was not specifically directed at diagnosis of premalignant lesions, as an initial positive sign for this approach, K-ras mutations were clearly visible in the 2 cases of ultimate malignancy, where the initial cytology was inconclusive and the clinical finding nonspecific. However, for more universal application it is expected that the required sensitivity is still below the current level. Therefore, for future diagnostic purposes it will be necessary to further increase the mutation detection sensitivity by alternative approaches such as pre-amplification and selective enrichment of mutants (9). Finally, it is expected that the 4 cases with inconclusive biopsy and no K-ras mutations were a result of the absence of ductal epithelia in the punctuate.

With the tumour-targeting, we expect to increase likelihood of finding also the less frequent mutations, thus broaden the range of potential diagnostic molecular markers to tumour-suppressor genes p16 and DPC. Naturally the genetic profiling of fine needle aspirates represents a significant diagnostic potential for patients with chronic pancreatitis, who would profit from regular dispensatory examination (laboratory, CT, endoscopic ultrasonography) and early surgery (21). A large control group of patients with chronic pancreatitis is necessary in order to evaluate the test specificity.

## Conclusion

We have demonstrated a pilot study on detecting K-ras mutations in FNA biopsy samples. The initial mutation frequencies were comparable to data published on mutation frequency found in pancreatic juice or pancreatic duct brushings, but lower when compared to equivalent FNA biopsy data. As a result, the methodology is to be modified, where instead of dissecting the biopsy sample for cytology and molecular-genetic evaluation, a tumour-targeting approach was applied in which DNA mutations were detected directly from cytology smears. We expect to broaden the current extent of the study to more patients and to add other prognostic markers in an ongoing continuation of this work.

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