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• CLINICAL RESEARCH •

# K-ras gene mutation in the diagnosis of ultrasound guided fine-needle biopsy of pancreatic masses

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## Abstract

**AIM:** To investigate the utility of K-ras mutation analysis of ultrasound guided fine-needle aspirate biopsy of pancreatic masses.

**METHODS:** Sixty-six ultrasound guided fine-needle biopsies were evaluated by cytology, histology and k-ras mutation. The mutation at codon 12 of the k-ras oncogene was detected by artificial restriction fragment length polymorphisms using *Bst* NI approach.

**RESULTS:** The presence of malignant cells was reported in 40 of 54 pancreatic carcinomas and K-ras mutations were detected in 45 of the 54 FNABs of pancreatic carcinomas. The sensitivity of cytology and k-ras mutation were 74 % and 83 %, respectively. The speciality of cytology and k-ras mutation were both 100 %. The sensitivity and speciality of k-ras mutation combined with cytology were 83 % and 100 %, respectively.

**CONCLUSION:** High diagnostic accuracy with acceptable discomfort of FNAB make it useful in diagnosis of pancreatic carcinoma. Ultrasound guided fine-needle biopsy is a safe and feasible method for diagnosing pancreatic cancer. Pancreatic carcinoma has the highest K-ras mutation rate among all solid tumors. The mutation rate of k-ras is about 80-100 %. The usage of mutation of codon 12 of k-ras oncogene combined with cytology is a good alternative for evaluation of pancreatic masses.

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## INTRODUCTION

Pancreatic adenocarcinoma is a very aggressive carcinoma and has the worst prognosis in common abdominal cancer[1-3]. Despite the poor prognosis, patients with localized disease may be cured with surgery[4-6]. However, it is difficult to diagnose pancreatic cancer in the earlier stages. This dismal prognosis is a result not only of biological aggressiveness but also of diagnosis late in the chronological progression of the tumor. If pancreatic cancer can be resected when it is small, the prognosis is much better, with a 5-year survival of approximately 40 %[7-9]. When pancreatic cancer is clinically suspected and a pancreatic mass identified by ultrasonography or computed tomography scan, a guided percutaneous fine-needle biopsy (FNAB) can be obtained; this may be the only sample available for diagnosis in most patients[10-12].

Even though alternative techniques for sampling cellular or tissue material have been developed, FNAB is still widely used for morphological verification of intra-abdominal malignancies, especially in pancreatic cancer. Although it has been questioned because of the risk of peritoneal seeding and seeding of tumor cells along the needle tract. It is still widely used for the diagnosis of pancreatic cancer combined with modern molecular biological techniques[13].

The high incidence of mutations at codon 12 of the K-ras gene (65-100 %) leads to consider them as a potential tumor marker at the tissue level[14-19]. The development of PCR-based techniques for detection of K-ras mutations has allowed its use in the clinical setting. The high incidence of mutation suggests that it may be used as a tumor marker at the tissue level. The role of k-ras detection in the clinical evaluation of pancreatic mass has to be established in a large series of patients. Data suggest that a combination of cytological examination and K-ras mutation detection in cellular material may improve diagnostic accuracy[20-26]. In this study we evaluated the diagnostic utility of cytological and histological examination and k-ras mutation detection, alone and in combination under the ultrasound guided FNAB from 66 patients with pancreatic masses.

## MATERIALS AND METHODS

### *Patients and samples*

Between January 1997 and May 2001, 66 consecutive patients (38 men and 28 women, mean age of 54±9 years) with pancreatic masses were included. In all cases FNAB of the masses were percutaneously obtained under ultrasonographic guidance. A portion (50 %) of each FNAB was immediately examined by pathologist. The other 50 % of the same specimen was frozen and stored in liquid nitrogen.

Final diagnosis of pancreatic carcinoma was established if malignant cells were identified in the FNAB or in surgically resected specimens or when death occurred within six months after diagnosis, with clinical evolution compatible with disseminated cancer disease. Other types of neoplasm were diagnosed on the basis of pathological findings. The diagnosis of chronic pancreatitis was based on standard clinical criteria. In chronic pancreatitis, a minimum of 6-month (range, 6-27 months) follow-up period with no evidence of cancer was available. Pancreatic tuberculosis was confirmed by positive Lowenstein culture.

### *Detection of K-ras codon 12 mutations*

DNA was extracted following standard procedures. We utilized

*Bst*NI (Promega, USA) restriction fragment length polymorphism/ polymerase chain reaction (RFLP/PCR) method that enriches for the amplification of mutant codon 12 K-ras alleles by cleaving amplified wild-type allele through intermediate digestion between first- and second-round PCR[27]. To create the restriction site for the enzyme *Bst*NI [CCTGG], which is lost when a K-ras codon 12 mutation exists, the first-round amplification was performed using the mutant primers K-ras 5' and DD5P (Table 1) in a volume of 50 μL containing PCR buffer (50 mmol/L KCl, 20 mmol/L Tris HCl, pH 8.4), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP (Promega, USA), 1U of Taq polymerase (Life Technologies, USA), and 150 ng of PCR primers[28]. The reactions were 10 cycles of 92 ℃(15s), 44 ℃(15s) and 72 ℃(15s). An aliquot of 5 μL of the amplified product was enzymatically digested with *Bst*NI following the manufacturer' s directions. One microliter of the digested product was reamplified using a heminested reaction with mutant amplimers K-ras 5' and K-ras 3' [28]. The reaction conditions were 35 cycles of 92 ℃(15s), 54 ℃(15s) and 72 ℃ (15s). The latter primer artificially introduces an internal control to assure the completion of enzymatic digestion. After polyacrylamide gel electrophoresis (6 %) and ethidium bromide (0.5 g/L) staining, the 143-bp band depicted the mutant allele, and the 114-bp band the wild-type allele. RFLP/PCR method consistently detected a mutant allele in serial dilutions containing at least 1 mutant allele in 1 000 wild-type alleles. Positive bands were always clearly identifiable when DNA obtained from FNAB was examined. All samples were analyzed in duplicate. Results were available 48-72 h after the tissue sample was obtained.

**Table 1** Primers for K-ras mutation detection

| Round | Primer | Sequence |
|---|---|---|
| First | K-ras 5' | 5'-ACTGAARARAAACTTGTGGTAGTTGGACCT-3' |
| | DD5P | 5'-TCATGAAAATGGTCAGAGAA-3' |
| Second | K-ras 5' | 5'-ACTGAATATAACTTGTGGTAGTTGGAACCT-3' |
| | K-ras 3' | 5'-TCAAAGAATGGTCCTGGACC-3' |

## RESULTS

### *Cytological examination*

Final diagnoses were as follows: 54 pancreatic carcinomas, 3 other malignancies (1 lymphoma, and 2 cholangio-carcinomas), 6 benign diseases (5 chronic pancreatitis and 1 tuberculosis), and 3 endocrine tumors. The presence of malignant cells was reported in 40 of 54 pancreatic carcinomas with no false positives. However, in 14 of the 54 FNABs of pancreatic carcinomas, the cytological report was not conclusive: 12 because of insufficient material and 2 because of suspicion. The sensitivity of cytology was 74 % in the diagnosis of pancreatic cancer.

### *Molecular diagnosis*

Molecular analysis was possible in 54 of 66 FNABs. K-ras mutations were detected in 45 of the 54 FNABs of pancreatic carcinomas, with no false positives. The combination of cytology and enriched RFLP/PCR analysis was always informative and showed a sensitivity of 83.3 %, with a specificity of 100 %. Only nine pancreatic carcinomas failed to be correctly classified after the combined cytological and molecular analysis.

In three cases, a K-ras positive analysis in combination with the presence of suspicious cells was considered confirmation of pancreatic cancer, and no further studies were performed. Two of these patients died 1 and 3 months later, respectively, with a clinical course consistent with advanced pancreatic cancer. In the other, positive peritoneal disease was present at surgery. In one patient with insufficient material at cytology, molecular analysis was the endpoint of the diagnostic work-up, and laparotomy was not performed because of the poor clinical status of the patient. Finally, in the remaining one patient with insufficient material for cytological evaluation and K-ras positive analysis, surgical resection of a histologically confirmed pancreatic carcinoma was performed.

## DISCUSSION

The influence of biopsy on the natural history of pancreatic carcinoma is still unclear, considering the increasing intention to treat and the development of new multimodality therapies[29,30]. Our data indicate the FNAB of pancreatic malignancy can be easily performed without serious side effects and is still a safe and useful procedure for establishing the diagnosis of pancreatic carcinoma. High diagnostic accuracy with acceptable patient discomfort has also been reported when using an 18-gauge cutting needle with an automatic spring-loaded sample device. The large amount of tissue obtained could improve the microscopic evaluation, but it has not been verified that microhistology offers advantages over cytology in the diagnosis of pancreatic cancer. The complication rate, including needle tract seeding, in pancreatic carcinoma is lower than in other tumors[31,32].

The main limitation of cytological analysis is the substantial proportion of cases in which a conclusive report is not possible, and where a second procedure to confirm diagnosis is required. FNAB can get a bigger tissues for microhistological examination. The molecular approach allows detection of K-ras mutants even when cells are present in a small proportion. Mutation detection would have complemented the cytological evaluation of FNAB when cellular material was insufficient, suspicious cells were present, or when healthy-appearing duct cells were reported[33].

Pancreatic carcinoma is known to have the highest K-ras mutation rate among all tumors. The codon 12 of this gene is affected in 80-100 % of the cases[34-36]. Only a minority of pancreatic cancers (9 cases) failed to be correctly classified by the combined histological and molecular approach in our studies. Although inaccurate sampling of the lesion may account for some of the false negatives observed, the molecular approach has some limitations[26,37]. The discrepancies between the results of the various studies are based on the wide range of investigated cases, the selection of lesion types, and the sensitivity of the microdissection and PCR techniques employed. The clinical usefulness of ras mutations relies on the development of rapid, sensitive, and reproducible techniques for their detection. Moreover, a positive molecular diagnosis avoided iterative pancreatic fine-needle aspiration or further diagnostic procedures in these patients.

In Japanese studies, a comparison of the K-ras mutation pattern in ductal lesions with that of the adjacent carcinomas revealed nearly identical mutation patterns[38,39], whereas in a study from North America a concordance rate of only 50 % was reported[40]. The results of our analysis showed identical mutation patterns in the primary tumor confirming the results of the Japanese authors. There are large differences in the incidence of ras mutations between Japanese and Western populations, although the reason is still unclear and the number of subjects is limited. In Japan, the frequency of K-ras mutation ranged from 55-80 %[41-44], whereas in West it was relatively low, ranging from 0-31 %[45,46]. The current data suggest that most patients have ras gene mutations in the tumor itself, which is similar to previous Japanese reports.

In conclusion, the results of the present study indicate that

K-ras analysis is a highly specific and sensitive approach in pancreatic carcinoma patients and suggest that a k-ras assay may have a role in the diagnostic assessment of these patients. Further investigations are needed to confirm these results, to improve the technique's sensitivity, and to establish its usefulness in the early.

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