Diagnosis of pancreatico-biliary malignancy: Detection of gene mutations in plasma and stool

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Summary
Chromosomal abnormalities, including mutations, deletions and allelic losses of different oncogenes and tumour suppressor genes have been discovered in the DNA of cancer cells and the application of molecular biological techniques now permits identification of these alterations in tumours. Although it has been possible to detect potentially important genetic alterations in tumour material for some years, it is now evident that many neoplasms shed tumour cells into sputum, urine, bile, pancreatic juice, faeces and blood of infected patients. Mutated DNA has also been detected free in the plasma of patients with cancer, and the DNA alterations in plasma are identical to those in the DNA of the primary cancer cells. Thus, the identification of DNA mutations in plasma, pancreatic juice and faeces might be a useful approach for the early detection and monitoring of patients with pancreatic cancer. The K-ras gene is mutated in over 90% of pancreatic cancer. These mutations are well defined, reliably detected by DNA application in assays and occur early in the genesis of pancreatic cancer. K-ras mutations can be detected in cancer tissue and pancreatic duct secretions. K-ras mutations have also been detected in stool of patients with pancreatic cancer. Invasive techniques for obtaining pancreatic juice or pancreatic tissue are undesirable and would certainly be inappropriate for cancer screening. Similarly, there is a lack of enthusiasm for developing diagnostic techniques that involve faecal extractions. Isolation of plasma DNA from pancreatic patients and detection of K-ras alterations with a PCR assay and subsequent product sequencing showed K-ras mutations in the plasma of 17 out of 21 patients (81%), and in cases in which both plasma and pancreatic tissue were available, DNA mutations were similar in plasma and tissue. Plasma DNA alterations were found 5-14 months before the clinical diagnosis of pancreatic cancer in 4 patients. K-ras mutations are also demonstrated in micro-dissected tissues taken from patients with pancreatic hyperplasia, with or without chronic pancreatitis. This has led to the suggestion that pancreatic cell hyperplasia may be a premalignant condition although the demonstration of K-ras alterations in some cases of chronic pancreatitis has raised doubts about the sensitivity and specificity of K-ras testing for pancreatic cancer. However, the detection of K-ras mutations in plasma may still identify patients with or at risk of developing pancreatic cancer as it may only be in these patients that sufficient quantities of mutated DNA enter and can be detected in plasma. Thus, this non-invasive approach to early cancer detection may be applicable both to diagnosis of the symptomatic patient and for screening. A combined approach with other tumour markers such as p53 gene might increase the sensitivity of the test.

Key words: DNA mutations, K-ras, p53, pancreatic cancer, PCR, plasma DNA, screening

Pancreatic cancer: Epidemiology and diagnosis
Pancreatic adenocarcinoma is the fourth most common cause of cancer death in the Western World with over 60,000 deaths in the United States and European Community each year. Potentially curative surgery is limited to small tumours without evidence of spread to other organs, but symptoms usually occur only at a late pathological stage. The median survival rate following diagnosis is therefore less than 6 months, and long-term survival rates are below 5%.

The poor survival following pancreatic cancer may be partly attributed to its biological aggressiveness and a relative lack of symptoms attributable to early disease. In addition, pancreatic adenocarcinoma can be difficult to distinguish from a number of other benign pancreatic conditions. Radiological and endoscopic investigations have simplified the diagnosis, but the clinical and radiological features are not specific to cancer. Thus, an ultrasound or CT guided fine needle aspiration or biopsy is often required to diagnose pancreatic cancer, but can yield a false negative result in 10%-40% of cases [1]. Biochemical markers have also been tested for their diagnostic potential, but no biomarker is currently sensitive or specific enough for routine clinical use.

Pancreatic cancer and K-ras gene mutations
The K-ras gene is mutated in 80-90% of pancreatic cancers. These activating mutations result in disordered proliferation and differentiation, are well defined and occur early in the genesis of this disease. Mutations associated with pancreatic cancer occur at codon 12 of the K-ras oncogene. They involve a single base pair change from GGT (glycine) to GAT (aspartic acid), CGT (arginine), GTT (valine) or TGT (cysteine), and are reliably detected by polymerase chain reaction (PCR) assays. Well characterised PCR assays for K-ras detection include amplification with mutation specific primers (MASA-PCR) [2]. Mutation-specific primers have 3' ends complementary to the individual mutation and, since Taq I polymerase lacks 3' exonuclease activity, it is unable to amplify DNA when the single base mismatch is located at the 3' end of the primer. This highly sensitive MASA-PCR assay can be optimised to detect K-ras mutations at a
From a diagnostic viewpoint, K-ras mutations have not only been found in pancreatic cancer tissues, but also in fine-needle aspirates, pancreatic juice, bile and duodenal aspirates [5-8]. These results, allied to the high incidence and early occurrence of K-ras alterations in pancreatic cancer, suggest that K-ras mutations might serve as a qualitative marker for this disease. However, a disadvantage of fine-needle pancreatic aspirates, pancreatic juice, bile and duodenal secretions is that they must be obtained either via transcutaneous puncture or at the time of endoscopy. Therefore, searches for K-ras mutations in faeces and blood have also been undertaken since they have the advantage of being obtained in a minimally invasive manner.

K-ras gene alterations in faeces of pancreatic cancer patients

K-ras gene mutations have been detected in the faeces of patients with colorectal cancer and in colonic adenomas. This indicates that oncogene DNA is shed into the alimentary tract and can survive in a non-denatured state to permit genetic analysis, including sequencing of the specific K-ras gene mutation. Caldas et al, analysed stool specimens using PCR and a plaque hybridisation assay in patients with pancreatic adenocarcinoma, cholangiocarcinoma and chronic pancreatitis. K-ras mutations were detected in 6 of 11 (55%) patients with adenocarcinoma, 2 of 3 (67%) patients with cholangiocarcinoma and 1 of 3 (33%) of patients with chronic pancreatitis [9]. In 5 of the 6 cases of pancreatic cancer the mutations in stool were identical to those in the tumour. Thus stool analysis for K-ras mutations has potential as a non-invasive approach to diagnosis although the inherent difficulties of handling stool specimens in a molecular genetic laboratory may be the reason why this technique has not been widely acclaimed and subjected to further evaluation.

K-ras gene alterations in circulating micrometastatic cells of pancreatic cancer patients

In 1993, Tada and colleagues first reported detection of K-ras mutations in micrometastatic cells obtained from the blood of pancreatic cancer patients [3]. Using the sensitive MASA-PCR technique, they detected mutations in 2 of 6 pancreatic cancer cases studied, but in none of 5 cases with insulinomas, pancreatitis or choledocholithiasis. This indicates that pancreatic cancers shed significant quantities of tumour cells into the bloodstream and, in some cases, certainly enough to be detectable above the enormous background ‘noise’ of wild-type DNA derived from leucocytes. PCR detection of micrometastases would be an attractive and minimally invasive method of detecting pancreatic cancer if these results were confirmed in larger studies. Indeed, Nomoto et al., using an RFLP-PCR assay, also detected K-ras mutations in circulating pancreatic cancer cells from blood samples taken between day 1 and 14 post laparotomy, but not in samples taken before operation [10]. This suggests that the detection of micrometastases in pancreatic cancer may be restricted to the intra-operative and early postoperative periods making it unsuitable for diagnostic purposes, at least with conventional PCR assays.

Plasma DNA in health and disease

In addition to its presence in leucocytes, viruses, bacteria and micrometastatic cancer cells within blood, strands of DNA are also found circulating free in blood plasma. Nucleic acids were first identified in human plasma by Mandel and Metais in 1948 [11]. However, it was not until the mid 1960’s that the clinical significance of circulating DNA was first appreciated, following the identification of DNA in the serum of patients with systemic lupus erythematosus (SLE) [12]. Researchers showed that DNA was also present in the serum and plasma of patients with other inflammatory diseases and in healthy subjects, usually at levels of approximately 10 ng/ml [13]. Further work using radioimmunoassays indicated that cancer patients tended to have even higher levels of circulating DNA, with levels above 1 μg/ml in some cases [14]. The identification of plasma DNA in cancer patients resulted in a number of quantitative studies investigating the relationship between plasma DNA levels and varied clinical and pathological features. Relatively high levels were associated with metastatic disease and the presence of disease which was unresponsive to therapy [15]. In addition, high levels were found to predict a poor prognosis [16] although this association was not sufficiently strong enough to be used in clinical practice.

Further progress in the field of plasma DNA and cancer awaited the development of a sensitive procedure for the extraction of minute quantities of DNA from plasma samples. Using a phenol, ether and chloroform extraction procedure followed by dialysis, removal of polysaccharide contaminants and lengthy centrifugation on a Cs2SO4 gradient [17], Stroun and colleagues initially extracted DNA from the plasma of patients with a variety of advanced malignant diseases. They also showed that plasma DNA was composed of double stranded fragments ranging up to 21 kb in length and was neoplastic in origin [17,18]. Plasma DNA extraction procedures have since become less complicated, and boiling techniques, co-precipitation with gelatin and DNA adsorption methods have all been used successfully to extract analysable quantities of DNA [19,20].

K-ras gene alterations in the plasma DNA of pancreatic cancer patients

A combination of relatively efficient extraction techniques with increasingly simple PCR methods resulted in studies on
mutations in DNA extracted from the plasma of cancer patients. The first PCR study was published in 1994 on N-ras mutations in haematological malignancies [21]. In that year the first study of mutant plasma DNA in pancreatic cancer was also published [22]. In this study, Sorensen et al. identified K-ras alterations in the plasma DNA of three pancreatic cancer patients, and identical mutations in tumour tissues from the two for whom tissue samples were also available. It was suggested that these markers might be useful for cancer diagnosis, determining response to treatment and the detection of early cancer recurrence.

Further work on plasma DNA alterations in pancreatic cancer was published in 1998. In a study by Mulcahy and colleagues [23], K-ras mutations were studied in plasma using an RFLP-PCR assay (see Figure 1).

![Figure 1](image)

Mutations were identified in the plasma of 17 of 21 cancer patients (81%) and, in cases where both plasma and pancreatic tissues were available, DNA mutations were similar in corresponding plasma and tissue samples. In contrast, mutations were not found in the plasma of two patients with chronic pancreatitis or in 5 healthy controls. Perhaps of greater interest, mutations were found in the plasma of 4 patients between 5 and 14 months later. The conclusion from this study was that a non-invasive, plasma based K-ras assay might provide qualitative diagnostic information to clinicians in the future.

A third study on plasma DNA K-ras alterations and pancreatic cancer was published by Yamada et al. in 1998 [24]. This group also detected K-ras alterations in plasma of 15 (60) cases in which a K-ras mutation was found in tumour tissue. These patients with plasma DNA mutations had larger tumours and were less likely to have a potentially curative procedure. In addition, treatment resulted in disappearance of K-ras gene mutations from plasma DNA in six of 9 patients (67%), while three patients with persistently positive plasma specimens had either an early recurrence or progressive disease. Other features, including patient age or sex, histological type, mode of invasion and metastasis did not correlate with the presence of mutated plasma DNA.

What are the implications of plasma DNA for clinicians interested in pancreatic cancer? Firstly, pancreatic adenocarcinoma can often cause diagnostic difficulty, and a reliable plasma based PCR assay would obviate the need for multiple biopsies or diagnostic laparotomy in a proportion of cases. Secondly, the association between mutant plasma DNA and tumour stage, the presence of residual disease and outcome suggests that it might potentially serve as a prognostic marker in the absence of clinically detectable metastases [24]. Perhaps more importantly, longitudinal measurements might be valuable in assessing a patient’s response to therapy and for the identification of local or distant disease recurrence at an asymptomatic and early stage [24].

The concept of a non-invasive plasma DNA test for pancreatic cancer diagnosis or screening is both attractive and aesthetically pleasing. However, the three studies performed to date on pancreatic cancer have studied only 45 cases in total, and further research is required to confirm the above results and to give a more accurate picture of the relationship between clinical and pathological features and the presence of mutant plasma DNA. A number of other questions also need to be answered before plasma DNA testing could be considered as a potentially useful tool for pancreatic cancer diagnosis.

Most researchers have been unable to detect K-ras alterations in non-neoplastic pancreatic tissues, indicating a high specificity of mutations for the presence of pancreatic cancer [3, 6-8, 23, 24]. However, Yanagisawa et al and Tada et al demonstrated K-ras mutations in microdissected tissues taken from patients with pancreatic cell hyperplasia, either in the presence or absence of chronic pancreatitis [25, 26]. Yanagisawa suggested that pancreatic cell hyperplasia may represent a premalignant condition analogous to the relationship between adenomatous polyp and adenocarcinoma in the large bowel [25]. However, the significance of this observation for plasma DNA testing remains unknown. It is possible that the presence of K-ras alterations in some cases of chronic pancreatitis may be important in elucidating pathways leading to pancreatic cancer, and that these mutations may be a predictor of future malignancy. It is also possible that K-ras alterations are not as specific for malignancy as most researchers think, and that its presence does not necessarily indicate the presence or imminent development of cancer. Further research on non-malignant pancreatic diseases would be valuable in order to determine the true specificity of plasma DNA K-ras mutations for cancer.

Other difficulties have also to be overcome before plasma DNA testing can be considered anything more than an interesting research phenomenon. The clinical value of any diagnostic test for cancer ultimately depends on its ability to detect either new or recurrent tumours. However, the K-ras gene is not mutated in all cases of pancreatic cancer, so that a single plasma based assay would lack a high sensitivity for disease. To improve detection rates, it may be necessary to analyse a more comprehensive panel of genes associated
with this disease. Furthermore, even if an adequate panel of genes is studied, it is probable that not all patients will have detectable levels of mutant DNA in their plasma. This has been the case in many studies on plasma DNA and cancer, but more sensitive techniques may allow even smaller quantities of mutant DNA to be characterised. Finally, there may be problems developing diagnostic and screening tests for early pancreatic cancer because the appearance of plasma DNA alterations tends to correlate with advanced disease [24,27]. This association is not precise, and mutant plasma DNA has been detected in the early stage of some cancers and in patients whose subsequent prognosis was good [27,28]. These uncertainties suggest that there is a need for further research to more clearly define the role of plasma DNA testing as a diagnostic and screening test for pancreatic cancer.

References


