Evaluation of clinical relevance of examining K-ras, p16 and p53 mutations along with allelic losses at 9p and 18q in EUS-guided fine needle aspiration samples of patients with chronic pancreatitis and pancreatic cancer

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CLINICAL RESEARCH

Abstract

AIM: To establish an optimum combination of molecular markers resulting in best overall diagnostic sensitivity and specificity for evaluation of suspicious pancreatic mass.

METHODS: Endoscopic ultrasound (EUS)-guided fine needle aspiration cytology (FNA) was performed on 101 consecutive patients (63 males, 38 females, 60 ± 12 years; 81 with subsequently diagnosed pancreatic cancer, 20 with chronic pancreatitis) with focal pancreatic mass. Samples were evaluated on-site by an experienced cytopathologist. DNA was extracted from Giemsa stained cells selected by laser microdissection and the presence of K-ras, p53 and p16 somatic mutations was tested by cycling-gradient capillary electrophoresis (CGCE) and single-strand conformation polymorphism (SSCP) techniques. In addition, allelic losses of tumor suppressor genes p16 (INK4, CDKN2A) and DPC4 (MADH4, SMAD4) were detected by monitoring the loss of heterozygosity (LOH) at 9p and 18q, respectively.

RESULTS: Sensitivity and specificity of EUS-guided FNA were 75% and 85%, positive and negative predictive value reached 100%. The remaining 26% samples were assigned as inconclusive. Testing of molecular markers revealed sensitivity and specificity of 70% and 100% for K-ras mutations (P < 0.001), 24% and 90% for p53 mutations (NS), 13% and 100% for p16 mutations (NS), 85% and 64% for allelic losses at 9p (P < 0.001) and 78% and 57% for allelic losses at 18q (P < 0.05). When tests for different molecular markers were combined, the best results were obtained with K-ras + LOH at 9p (92% and 64%, P < 0.001), K-ras + LOH at 18q (92% and 57%, P < 0.001), and K-ras + LOH 9q + LOH 18q (96% and 43%, P < 0.001). When the molecular markers were used as complements to FNA cytology to evaluate inconclusive samples only, the overall sensitivity of cancer detection was 100% in all patients enrolled in the study.

CONCLUSION: EUS-guided FNA cytology combined with screening of K-ras mutations and allelic losses of tumor suppressors p16 and DPC4 represents a very sensitive approach in screening for pancreatic malignancy. Molecular markers may find its use particularly in cases where FNA cytology has been inconclusive.

Key words: Pancreatic cancer; Chronic pancreatitis; Endoscopic ultrasound-guided fine-needle aspiration; Molecular markers; Loss of heterozygosity

INTRODUCTION

Enormous progress in diagnostic and therapeutic approaches in the last decade had very limited impact on generally poor survival rates of patients diagnosed with
pancreatic cancer (PC)\cite{1}. Mortality of the disease is almost at the level of its incidence as the majority of cases are diagnosed in advanced, not resectable stage\cite{2}. Since the fundamental molecular-genetic mechanisms of PC have already been recognized, there is a great expectation that molecular tests could substantially assist in early diagnosis as well as open new therapeutic possibilities for this serious disease\cite{3,4}.

The development of pancreatic cancer follows a distinct path from normal ductal epithelia, pancreatic intraepithelial neoplasia (PanIN 1-III) up to the carcinoma\cite{5,6}. This path is accompanied by sequential accumulation of genetic defects (mostly point mutations, gene amplifications and allelic deletions). Activation of K-ras oncogene by somatic point substitution is seen as an initial event in pancreatic carcinogenesis\cite{7}. This alteration can be detected already in PanIN-1A lesions as well as in chronic pancreatitis (CP) and therefore represents an independent risk factor for pancreatic cancer. In advanced pancreatic cancer, K-ras mutations are found in close to 90% of cases, therefore considered as a potential molecular marker for early detection of developing cancer. 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. Increased Her-2/neu expression, however, is a result of higher transcription rate rather than gene amplification, rendering Her-2/neu an unusable therapeutic target\cite{8}.

Aside from the above oncogenes, there are a number of tumor suppressor genes affected by genetic alterations during the transformation process. Among them, p16 tumor-suppressor (also referred to as CDKN2 or INK4), located at chromosome 9p21 is inactivated already during transition from PanIN-1B to PanIN-2 phases\cite{9}. Furthermore, loss of another important tumor-suppressor gene, SMAD4 (known also as deleted in pancreatic carcinoma, DPC4), located at chromosome 18q21 has also been observed\cite{10}. Consequently, the p16 and DPC4 are inactivated in almost 95% (55% respectively) of cases of invasive pancreatic cancers, therefore, potentially useable as molecular markers. All of the genetic mutation events adversely affect control of the cell cycle, thus enabling defective cells to proliferate. Oncogene K-ras encodes for GTP-binding protein responsible for signaling in the MAP-kinase pathway of intracellular signal transduction\cite{11}. Tumor suppressor gene p53 is translated into a protein that regulates transcription of other regulatory proteins, such as p21, inhibitory protein of cyclinD/CDK2 family\cite{12}. The product of p16 tumor suppressor binds to the complex of cyclinD/CDK4 or CDK6, and thus regulates progression of cell cycle at the G1 control point\cite{13}. Finally, the DPC4 tumor suppressor is a member of the SMAD protein family which plays a crucial role in intracellular signaling of TGF-beta\cite{14}.

Current diagnostic approaches mostly rely on evaluation of morphological changes in pancreatic tissue in combination with histology/cytology examination of samples obtained by fine-needle aspiration (FNA)\cite{15}. EUS-guided FNA typically delivers sensitivity of 80% and specificity of 99%, while its positive and negative predictive values are at 99%, and 73% levels\cite{16}. In order to increase diagnostic sensitivity of the FNA cytology, several papers have demonstrated detection of somatic aberrations as potential markers for early pancreatic cancer in DNA material from pancreatic juice, pancreatic ductal brushings, perioperative or percutaneous biopsies, plasma, duodenal aspirate, bile or stool. Among the various molecular markers in pancreatic cancer, K-ras is the most frequently studied. Its prevalence is estimated to reach 90%-95%. The reported rates of positivity, however, depend on experimental method of K-ras mutation detection as well as on the source material in which presence of K-ras mutations is to be detected. The capture rates range from 78%-100% in pancreatic tissue\cite{17}, 61%-89% in pancreatic juice\cite{18,19}, 72%-83% in pancreatic ductal brushing\cite{20,21}, 35% in plasma\cite{22}, 33% in bile\cite{23}, and 25% in duodenal aspirate\cite{24}. Detection of K-ras in stool gives better sensitivity than in bile, however specificity drops significantly\cite{25}. Acceptable specificity was reported only in pancreatic ductal brushings and pancreatic juice (77%-100%).

Because of the high sensitivity of genetic testing in pancreatic juice, numerous mutations in other genes have been reported in this material. Sensitivity and specificity of genetic tests in pancreatic juice is 40%-89% and 33%-96% for K-ras\cite{18,19}, 11%-43% and 70%-100% for p16\cite{18,26,27,28}, 14%-47% and 88%-100% for p53\cite{29,30}, 36%-70% and 39%-100% for DPC4\cite{18,30}. The combination of several molecular markers in pancreatic juice is believed to improve sensitivity of genetic testing, giving best results for combination K-ras plus p53 which resulted in 100% sensitivity\cite{25}. In contrast to pancreatic juice analysis, there are only a limited number of publications on frequency of gene mutations in EUS-guided FNA samples. Takahashi's study which included 62 consecutive patients with focal pancreatic mass is the largest. The authors screened for K-ras mutations and gave 74% sensitivity with 100% specificity\cite{31}.

From the original PC progression model it is clear that the pancreatic malignant conversion comes from a combination of multiple genetic events rather than originating from a single mutation\cite{32}. Given the inherent heterogeneity of the carcinogenic pathways, simultaneous examination of multiple markers should lead to improved testing efficacy. The aim of the presented work was to evaluate a possibility of examining several somatic genetic events as potential molecular markers for early detection of pancreatic cancer in risk groups, such as in chronic pancreatitis patients. The main emphasis was on finding an ideal combination of markers resulting in optimum results when used in combination with commonly used cytology readings.

**MATERIALS AND METHODS**

**Subjects**

A total of 106 consecutive patients with focal pancreatic mass undergoing EUS-guided fine needle aspiration (FNA) were enrolled into the study. Patients were divided into pancreatic cancer group and the control group of patients with chronic pancreatitis based on histology of

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surgical specimen or long-term follow-up. Five patients were excluded due to other diagnosis (adenoma, malignant fibrous histiocytoma, endocrine tumor, cholangiogenic carcinoma), or for malignant duplicity (patient with a breast carcinoma).

Of 101 patients in the final group a total of 63 (62%) were males and 38 (38%) females. The mean age in the group was 60 ± 12 years, range 32-84 years (+2.01/-2.21 standard deviation). There were 81 (80%) patients with pancreatic cancer, and 20 (20%) patients with chronic pancreatitis. All patients signed informed consent with participation in the study as well as with genetic analysis of their tissue material.

**Methods**

EUS was performed by a single experienced endoscopist using GFUM-20 radial and GFUCT-140 linear array scanning echoendoscopes (Olympus Europe). Quality of FNA samples was evaluated by an on-site cytologist after quick staining by hematoxylin-eosin. Definitive FNA diagnosis was stated by a single pathologist, blinded to the EUS, after staining additional slides by Giemsa. The same samples were subsequently submitted for genetic analysis. Furthermore, in a subset of 18 patients the genetic analysis of FNA samples were extended to genetic analysis of tissue acquired during subsequent perioperation biopsy. Laser microdissection of Giemsa-positive cells was performed on a P.A.L.M. Microlaser instrument (Carl Zeiss, Germany). Normally, between 100 and 200 cells were dissected from each slide. Genomic DNA was extracted from the dissected cells by a standard spin-column extraction protocol using the GMC tissue DNA isolation kit (Genomac, Czech Republic).

Presence of somatic point mutations in codons 12 and 13 of K-ras and in exons 5-8 of p53 was detected by cycling-gradient capillary electrophoresis (CGCE), a high-sensitivity mutation detection technique based on heteroduplex analysis in a temperature gradient [32]. The experimental details of the K-ras and p53 mutation assay were described previously [33,34]. Briefly, a PCR amplification of the target sequence containing the mutation hotspots was performed with one of the primers fluorescently labeled and the other primer extended by a 40 bp artificial high-melting domain (GC-clamp). Following PCR, the 140 bp fragment was heated and slowly cooled to allow formation of homo- and heteroduplex forms upon re-annealing of wildtype and mutant sequences. The resulting double-stranded fragments were subjected to capillary-electrophoretic separation at a cycling temporal temperature gradient. A typical result is shown in Figure 1.

Mutations in the p16 gene were analyzed by standard single-strand conformation polymorphism (SSCP) using amplification conditions previously described in literature [35,36] followed by capillary electrophoresis analysis of the resulting fragments in a non-denaturing gel matrix (GMC-SSCP, Genomac, Czech Republic).

Allelic losses at chromosomal positions 9p and 18q were monitored by the loss of heterozygosity analysis (LOH) using a total of 3 microsatellite (STR) markers for 9p (D9S157, D9S171 and D9S1748) and 2 markers for 18q (D18S363, D18S474) [37,38]. Detected LOH at chromosomal site 9p is shown in Figure 2.

All capillary electrophoretic experiments including previously described temperature-gradient, SSCP and LOH analysis were performed on a capillary-array DNA sequencer (MegaBACE™ 1000, GE Healthcare, Piscataway, NJ) equipped with Caddy™ 1000 robotic sample loader (Watrex Praha, Prague, Czech Republic) for unattended overnight operation.

**Statistical analysis**

Analysis was based on two-way and multiway contingency tables, sensitivity and specificity of tests and on a 95% confidence interval of relative frequencies with use of BMDP PC90 and MedCalc software.

**RESULTS**

The data in the study represent patients collected within a 2-year period from 2003 to 2005. Due to the dismal nature of the disease the total project time period exceeded by far the mean survival rate of pancreatic cancer patients enrolled in this study. The ultimate diagnosis of the malignant disease could, therefore, be unequivocally assigned based on clinical follow-up. During the final statistical analysis and evaluation, sensitivity and specificity of various diagnostic approaches performed during the patients dispensation could later be accurately determined.

**Endoscopic ultrasonography (EUS)**

EUS is considered the most sensitive method for visualizing...
focal pancreatic lesions and staging of locoregional progression of the pancreatic disease. All patients in our group were subjected to EUS for initial evaluation of pancreatic lesions. The EUS differentiation between the malignant or benign nature of the lesions resulted in 79% sensitivity and 77% specificity. The overall rate of false negatives was 5% and false positives 4%. In 11% of cases the endoscopist was not able to reliably state the diagnosis.

**Fine-needle aspiration cytology (FNA-cytology)**

EUS-guided FNA cytology has been adopted as a routine method for all patients admitted to our gastroenterology department with a suspicion of pancreatic cancer. The main benefit of this safe method is in its sensitivity. At the same time, the acquired morphological information (TN staging) removes a need for additional diagnostic testing and/or surgery, surpassing CT or MR imaging.

Following the initial test for specimen quality by a “quick-test” using hematoxylin-eosin staining immediately following the puncture, samples were stained by Giemsa and thoroughly evaluated by an experienced cytopathologist. In the present study the overall sensitivity and specificity for FNA cytology evaluation was 75% and 85% respectively; the positive and negative predictive value reached 100% with no malignant specimens assigned as benign or vice versa. In FNA testing, however, 26% of smears were assigned as inconclusive. This mostly owing to the fact that the cellular atypia found in ductal epithelia did not allow clear differentiation between both diagnoses.

**Histology of surgical resection tissue**

Finally, in a subset of 18 patients, surgery was performed and collected pancreatic tissue was evaluated by a pathologist. Histological evaluation of surgical resection resulted ultimately in 100% specificity, but 95% sensitivity due to the fact that one resection sample was falsely evaluated as cancer-negative.

**Molecular marker examination**

Activating mutations in the K-ras oncogene were found in 57 out of the total 101 samples. Comparison with the final diagnosis revealed that all K-ras positives were subsequently confirmed with malignancy, while none of the chronic pancreatitis samples exhibited K-ras mutation. Hence, the resulting specificity was 100% and the sensitivity 70% with 95% CI (60%-80%). There were 24 (30%) cancerous specimens without K-ras mutation. Detecting mutations in tumor suppressor gene p53 uncovered only 19 positive cases (total of 101 cases) with a sensitivity of 24% with 95% CI (14%-32%) and specificity of 90% with 95% CI (85%-95%). Similarly, low mutation rates were obtained for p16 gene with 10 of 100 cases leading to only 13% sensitivity (95% CI 5%-9%) and specificity of 100%. Forty-four of a total of 66 samples exhibited allelic loss at 9p with a sensitivity of 85% with 95% CI (75%-94%) and specificity of 64% with 95% CI (53%-75%). Although 9p harbors p16 tumor-suppressor gene, no correlation was found between occurrence of p16 mutations and 9p allelic deletions. A combination of the two tests (p16 mutations and losses at 9p) yielded overall sensitivity of 84% with a specificity of 64%, 95% CI (75%-94%) and (53%-75%) respectively. Sole LOH test at chromosomal position 18q, corresponding to a loss of tumor suppressor gene DPC4, was detected in 38 of 63 cases with a sensitivity of 78% (95% CI 66%-89%) and specificity of 57% (95% CI 45%-69%) (Table 1).

When combining tests for independent molecular markers (Table 2), the best results were obtained with a combination of K-ras and LOH 9p. Sensitivity and specificity of this combination were 92% with 95% CI (86%-99%) and 64% with 95% CI (53%-75%), respectively. Another promising combination was K-ras and LOH 18q resulting in sensitivity of 92% with 95% CI (85%-99%) and a specificity of 57% with 95% CI (45%-69%). By combining two markers with high specificity K-ras and low-sensitivity p53 reasonable values were obtained: sensitivity of 74% with 95% CI (65%-83%) and specificity of 90% with 95% CI (85%-95%). This, however, does not significantly improve the sole K-ras test showing 70% sensitivity and 100% specificity as noted above. Similarly,
DISCUSSION

EUS-guided FNA-cytology is widely regarded as the “golden standard” in morphological diagnosis of pancreatic neoplasms. In agreement with this common perception, our own experience also confirms a high diagnostic value of the technique with positive and negative predictive value reaching 100% over the course of the presented study. These results mirror high efficacy of the protocol if FNA biopsy is first evaluated on-site by the cytologist, and then conclusively interpreted by a skilled pathologist with proper experience in pancreatic cytology.

Table 2  Combination of test for various molecular markers

| Combination of tests | 95% CI     | P        | Youden's index |
|----------------------|------------|----------|----------------|
| K-ras + LOH 9p       | Sensitivity 92% | 85%-99% | < 0.001 | 57% |
|                      | Specificity 64% | 53%-75% |        |      |
| K-ras + LOH 18q      | Sensitivity 92% | 85%-99% | < 0.001 | 49% |
|                      | Specificity 57% | 45%-69% |        |      |
| LOH 9p + LOH 18q     | Sensitivity 92% | 85%-99% | < 0.01  | 34% |
|                      | Specificity 43% | 31%-55% |        |      |
| p16 + LOH 9p         | Sensitivity 84% | 74%-94% | < 0.001 | 49% |
|                      | Specificity 64% | 53%-75% |        |      |
| K-ras + p53          | Sensitivity 74% | 65%-83% | < 0.001 | 64% |
|                      | Specificity 90% | 85%-95% |        |      |
| K-ras + LOH 9p + LOH 18q | Sensitivity 96% | 92%-100% | < 0.001 | 39% |
|                      | Specificity 43% | 31%-55% |        |      |
| K-ras + p16 + LOH 9p + LOH 18q | Sensitivity 92% | 85%-99% | < 0.001 | 57% |
|                      | Specificity 64% | 53%-75% |        |      |
| p53 + LOH 9p         | Sensitivity 92% | 85%-99% | < 0.001 | 35% |
|                      | Specificity 43% | 31%-55% |        |      |

This encouraging result, however, is reduced by the fact that in addition to the unequivocally assigned samples a remaining total of 26% of FNA smears are marked as inconclusive. This mirrors the fact that distinction between reactive changes in chronic pancreatitis and well differentiated adenocarcinoma may be problematic and cause under diagnosis of pancreatic cancer.[41] Hence, the resulting 74% success rate of FNA-cytology clearly opens a need for additional diagnostic tools.

Molecular diagnosis of early pancreatic cancer has been studied for several years. Although many molecular markers have been identified, it is evident that diagnostic and/or screening should be based on a set of tests rather than relying on one universal molecular indicator. In our study, we have obtained reproducible results indicating a notable capture rate of pancreatic cancers by using a combination of highly specific multiple markers. As shown in Table 1, the test for K-ras mutation exhibited the highest possible specificity. Our finding is in agreement with reports of K-ras testing in pancreatic juice (sensitivity of 40%-89% and specificity 33%-96%). With regard to K-ras testing in FNA samples, our sensitivity was similar to a recent study (70% vs 74%), moreover, at the same time we have confirmed 100% specificity of K-ras testing in FNA reported in the same study[41]. The fidelity of the K-ras test in our work was followed by LOH analysis for the detection of allelic losses at 9p and 18q chromosomal positions. Satisfactory sensitivity with relatively low specificity of all above mentioned genetic tests make them suitable for screening purposes rather than for differential diagnosis of pancreatic masses.

As expected, the LOH analysis greatly profited from laser microdissection of tumor cells from FNA-cytology specimens. In comparison to manual dissection from resected tissue, the sensitivity for detection of allelic loss was higher for laser-microdissected FNA samples. Low diagnostic value of p53 and p16 point mutations is in agreement with the overall limits of sensitivity and specificity intervals for these markers being previously tested in pancreatic juice.[10,25,27-29] Similarly in FNA samples, p53 or p16 mutations seem suitable for differential diagnosis or screening in FNA samples.

Based on the observations from this study, a diagnostic algorithm reflecting the most efficient approach to distinguish pancreatic cancer from chronic pancreatitis in FNA samples can be constructed (Figure 3). As the EUS-guided FNA-cytology still has the highest diagnostic relevance reaching 100% both predictive values while showing acceptable sensitivity and specificity, it should always remain a preferred method of choice for examination of a focal pancreatic mass (Figure 3, step 1). Only a subset of FNA-inconclusive samples should be further examined by genetic analyses. The size of such a sample set will undoubtedly depend on the pathologist's level of expertise. Of the various markers, K-ras is a prime candidate for first-level genetic analysis as the K-ras positivity showed to reliably differentiate patients with malignancy (Figure 3, step 2). Because of a lower sensitivity of the K-ras test, samples negative for K-ras mutation should, consequently, be examined for allelic losses by LOH tests. Due to its higher sensitivity, LOH
on the chromosome 9p, should be tested first (Figure 3, step 3), followed by a final testing of the LOH 18q performed on the remaining samples showing negativity for all previous tests (Figure 3, step 4). Such a set of four subsequent testing steps delivers satisfactory results. When using to process data acquired in our study, malignancy was correctly assigned to all patients with ultimately confirmed cancer status with no false negatives. One patient with chronic pancreatitis was incorrectly assigned with pancreatic cancer, a false positive, due to positivity of both 9p and 18q LOH tests.

In conclusion, the most sensitive genetic test for screening for malignancy in EUS-guided FNA samples from pancreatic mass seems a combination of K-ras mutation analysis with detection of p16 gene loss by LOH at 9p. Combination of K-ras with LOH analysis at both p16 and DPC4 genes further improves the sensitivity to 96%. The best compromise of sensitivity and specificity according to the Youden's index is single K-ras (70%) or combination of K-ras with LOH 9p (57%). Based on our observations it seems that due to relatively high specificity of the used markers, malignancy is usually indicated already by a single positive test. Therefore, only negative samples are subsequently tested by further markers, increasing the cost effectiveness of such diagnostic testing.

COMMENTS

Background
According to the numerous papers published in recent years, molecular diagnostics do not exceed the capabilities and limitations of conventional histological or cytological procedures. However, its contribution to the diagnostics in cases where morphology is not conclusive was not taken into account.

Research frontiers
The development of a diagnostic approach to pancreatic cancer relying on the analysis of genetic markers is a hotspot for scholars for the past decade. As monitoring of singular molecular markers has failed in this endeavor up to date, the focus has shifted to expression profiling, proteomics, and development of diagnostic arrays.

Innovations and breakthroughs
The study applies genetic methods previously tested in pancreatic juice directly on DNA extracted from tumour cells won by EUS-guided FNA. It was performed in a relatively large group of patients (n = 101) and monitors changes in four genetic loci (K-ras, p16, p53 and DPC4). No previous study describing loss of heterozygosity at 9p and 18q in pancreatic FNA samples was published up to date.

Applications
A sequential four-step diagnostic algorithm combining EUS-guided FNA and various genetic tests is suggested for differentiating between benign and malignant focal pancreatic masses. Its potential to become a part of a screening program for pancreatic cancer in high risk groups of patients is to be evaluated.

Terminology
Endoscopic ultrasound navigated fine needle aspiration (EUS-guided FNA) is a safe and highly sensitive method for getting pancreatic tissue samples by transgastric or transduodenal puncture. Fine needles (17-19 Gauche) allow obtaining high quality material for cytology examination which, however, is not generally sufficient for biopsy samples. Cycling gradient capillary electrophoresis (CGCE) is a high-sensitivity mutation detection technique. PCR fragments are heated and slowly cooled so that homo- and heteroduplex forms arise upon re-annaling of wildtype and mutant sequences. The resulting double-stranded fragments are separated according to different velocity in capillary electrophoresis at cycling temperature gradient.

Peer review
The authors evaluated clinical relevance of multiple genetic alterations in EUS-guided fine needle aspiration samples of patients with chronic pancreatitis and pancreatic cancer. The data are very informative because the experiments are well designed and performed.

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