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Differential Diagnosis of Pancreatic Tumors by Molecular Analysis of Clinical Specimens

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Key Words
Pancreatic cancer · Differential diagnosis · Molecular markers · Multiplex assays

Abstract
Objective: To describe the capabilities and limitations of the current state of the art in pancreatic cancer diagnostics and to discuss recent progress in the development of novel, highly accurate molecular diagnostic approaches. ***Results:*** Molecular analyses currently under evaluation as novel diagnostic tests include detection of point mutations, genomic imbalances, aberrant methylation patterns and gene expression changes on the mRNA and protein levels in pancreatic juice, fine needle aspiration biopsies and brush cytologies. ***Conclusions:*** In exploratory studies, several candidate molecular markers show great potential to serve as general indicators of malignancy, but need to be validated in large, controlled, prospective studies. Multiplexing of diagnostic tests, e.g. in the form of specialized DNA microarrays, may provide more differentiated diagnoses such as the distinction of various tumor types or prognostic information for individual patients. The MolDiag-Paca consortium is strongly engaged in advancing these developments on a European level.

Introduction

Pancreatic ductal adenocarcinoma (PDAC), which accounts for more than 90% of all malignant tumors in the pancreas [1], carries the most dismal prognosis of all solid tumors [2]. At present, surgical resection of early stage tumors is the only available potentially curative treatment option. However, both the timely detection as well as the accurate differential diagnosis of pancreatic cancer remain exceedingly difficult with currently available diagnostic means. When patients present with a suspect mass in the pancreas, accurate differentiation between malignant and benign processes of the pancreatico-biliary system is critical to the provision of adequate treatment. Approximately 10% of the pancreaticoduodenectomies performed for presumed malignancy reveal benign disease on pathological evaluation [3, 4]. Conversely, false-negative diagnoses may result in unnecessary and potentially harmful delay in the treatment of malignant tumors. In addition to the general distinction between malignant and benign processes, accurate differentiation between PDAC and other pancreatico-biliary malignancies is important because all other tumor entities have significantly better prognoses and may require profoundly different pre- and postoperative treatment.

The availability of highly sensitive and specific (as well as cost-effective) diagnostic procedures would also be a

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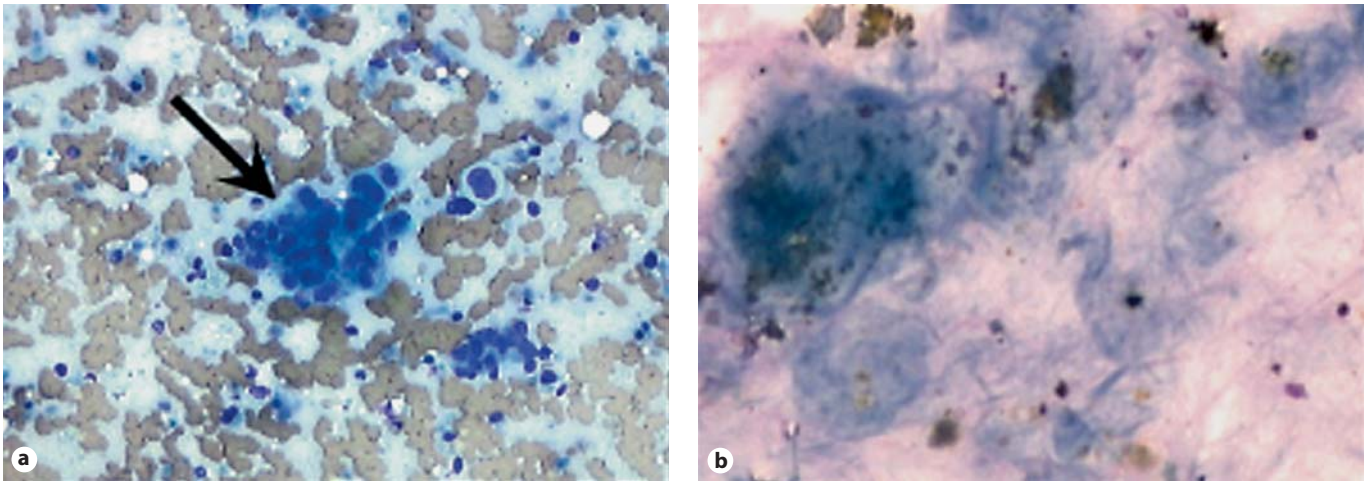

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Fig. 1. Typical examples of a clearly malignant (**a**) and a nondiagnostic (**b**) finding at cytological analyses of FNAB samples. **a** Malignant cells (arrow) showing nuclear overlapping, irregular contours, high nuclear/cytoplasmic ratios and anisocaryosis are readily detectable. **b** The specimen contains large amounts of mucus and cellular debris with few or no intact cells. Reproduced from Buchholz et al. [86].

prerequisite for the implementation of screening programs for early pancreatic cancer. As mentioned above, only the detection of very early stages of PDAC offers any chance for curative treatment [5]. However, due to the typical late onset and unspecific nature of the symptoms, at present only 10–15% of patients will be amenable to potentially curative resection at the time of diagnosis [6]. While screening of the general population does not seem feasible in the foreseeable future due to the relatively low prevalence of the disease, the discovery of novel molecular markers of pancreatic cancer may pave the way to routine screening of high risk groups such as persons suffering from Peutz-Jeghers syndrome (up to 132-fold risk [7, 8]) or long-standing chronic pancreatitis (15- to 25-fold increased risk [9]) as well as persons with a family history of hereditary pancreatitis (70-fold risk [10]), familial atypical multiple mole melanoma (13- to 65-fold risk [11, 12]) or familial pancreas cancer (32-fold risk [13, 14]).

To date, diagnosis (as well as staging) of pancreatic masses mainly relies on a variety of imaging techniques. These include ultrasound followed by multi-detector row computed tomography, and endoluminal ultrasound (EUS). Additional options include endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance imaging with magnetic resonance angiography, magnetic resonance cholangiopancreatography and positron emission tomography (PET) with or without simultaneous CT (PET/CT). Normally, combinations of different imaging modalities are employed in the preoperative diagnosis and staging of patients with suspected pancreatic carcinoma, since no single technique provides sufficient sensitivity and specificity by itself.

Due to the obvious limitations of the different imaging techniques, samples for cytopathological or histological assessment are often obtained to help confirm the diagnosis of a suspected malignancy. Samples can be obtained endoscopically by EUS and ERCP or percutaneously under CT or ultrasound control. In the cytopathologic evaluation of the biopsy material, diagnostic criteria for malignancy are very well established and include nuclear enlargement, pleomorphism (minimum of 3- to 4-fold variation in nuclear size), elevated nuclear/cytoplasm ratio, nuclear membrane irregularity, and coarse chromatin [15]. The specificity of diagnosis using these criteria is extremely high and routinely approaches 100% [15–18]. However, problems and inconsistencies arise if the sampled cells do not display all criteria of malignancy or if the cellular content of the sample is too low (fig. 1). As a result, between 11 and 30% of samples obtained by endoscopic ultrasonography-guided fine needle aspiration biopsy (EUS-FNAB) have to be classified as nondiagnostic (inadequate or equivocal diagnoses) [15, 18–20], severely reducing the sensitivity of the procedure. The same is true for the evaluation of endoscopic brush cytologies obtained at ERCP, which is the method of choice for pursuing tissue diagnosis in patients with pancreato-biliary strictures. Here, the reported sensitivity ranges between 30 and 80%, with an average of 68% [21–24].

Molecular Markers of Malignancy

The obvious limitations of conventional diagnostic procedures in the detection and classification especially of small pancreatic lesions has spurred the search for additional molecular markers with the potential to increase the sensitivity and specificity of diagnosis. It is well established that the process of cancerogenesis in the pancreas is associated with the accumulation of characteristic genetic changes within the cells of origin. Among the hallmark features of PDAC are mutations in the *K-ras* and *HER2/neu* oncogenes as well as the *p53*, *p16INK4a* and *SMAD4/DPC4* tumor suppressor genes [for an overview, see 6, 25]. More recently, high-throughput screening analyses have identified a multitude of differences between malignant and benign processes in the pancreas, as well as between different malignant tumor entities, on the genome [26–29], transcriptome [30–43], proteome [44–48] and epigenome [49–52] levels. Based on these results, a growing number of studies report on the use of one or several of these molecular markers in diagnostic analysis of serum, pancreatic juice, brush cytologies or FNABs.

Initially, most of these studies were aimed at detecting mutant *K-ras* in biopsy samples, since this is the gene most frequently affected by mutations (>85% of cases) in PDAC. Results were disappointing, however, since *K-ras*-mutations were also detected in up to 25% of samples from chronic pancreatitis patients [53, 54] and even in healthy pancreata from elderly control subjects [55], severely compromising the specificity of the tests. Detection of *K-ras* codon 12 mutations does, however, prove useful when combined with other molecular diagnostic tests. Yan et al. [56] demonstrated that the combined analysis of K-ras mutation status, p53 mutation status and $p16^{INK4a}$ promoter methylation status strongly increases the power to discriminate between malignant and benign disease. Their calculations suggest that this combination of tests may be accurate enough to be useful for the stratification of high-risk populations. Salek et al. [57] analyzed the same set of mutations, augmented by detection of allelic losses of the p16 and SMAD4/DPC4 gene loci, in EUS-FNAB samples and reported a sensitivity and specificity of 100% when the molecular tests were combined with conventional cytology. In a series of studies, Khalid and coworkers combined the analysis of K-ras codon 12 mutations with loss of heterozygosity analyses of panels of tumor suppressor-linked microsatellite markers to evaluate biliary brush cytology samples [58], pancreatic cyst fluid [59] and EUS-FNAB samples [60]. Remarkably, their analyses yielded 100, 92 and 100% diagnostic accuracy, respectively, for the limited study populations investigated.

A promising novel candidate to serve as a molecular marker of malignancy is the *KH homology domain containing protein overexpressed in cancer* (KOC), also known as *insulin-like growth factor 2 mRNA-binding protein 3*. KOC is an oncofetal RNA-binding protein that seems to be involved in the posttranscriptional regulation of cell proliferation during embryogenesis [61]. It is strongly overexpressed in pancreatic cancer [62], while its expression in normal adult tissues appears to be limited to the placenta [63]. In a study by Mueller et al. [64], detection of KOC mRNA by RT-PCR in a series of 41 FNAB samples from different abdominal lesions resulted in a sensitivity and specificity for the detection of malignancy of 93 and 83%, respectively. In a subsequent study examining immunohistochemical KOC staining patterns in surgically resected pancreatic tissues, strong staining was observed in 97% of invasive carcinomas (PDAC, papillary-mucinous carcinomas and mucinous cystadenocarcinomas) as well as the majority of advanced PanIN lesions, while staining was weak or absent in all benign cells and tissues [65]. Finally, a recent study by Zhao et al. [66] on immunohistochemical detection of KOC expression on EUS-FNAB samples demonstrated a sensitivity and specificity of 88 and 100%, respectively, for the KOC immune staining alone, and 95 and 100% for the combination of cytology and KOC staining.

Another candidate with high diagnostic potential is S100P, a member of the S100 family of calcium-binding proteins. S100P has been reported to be overexpressed in pancreatic cancer as compared to chronic pancreatitis and normal pancreas in several independent microarray studies [34, 35, 67–69]. S100P is of special interest, since in a microarray study analyzing microdissected pancreatic tissues, it was found to be not only overexpressed in invasive carcinomas, but also in precursor lesions of pancreatic cancer [36]. Similarly, immunohistochemical analysis of a series of pancreatic cancer and PanIN tissues demonstrated S100P to be expressed in 31% of PanIN-2 lesions, 41% of PanIN-3 lesions, and 92% of invasive PDAC specimens [70]. Together, these results suggest that S100P may be suitable to serve as an early marker of malignancy. A recent study on the immunohistochemical detection of S100P in FNAB samples [71] as well as one study on the detection of S100P mRNA in pancreatic juice [72] support the notion that this gene may emerge

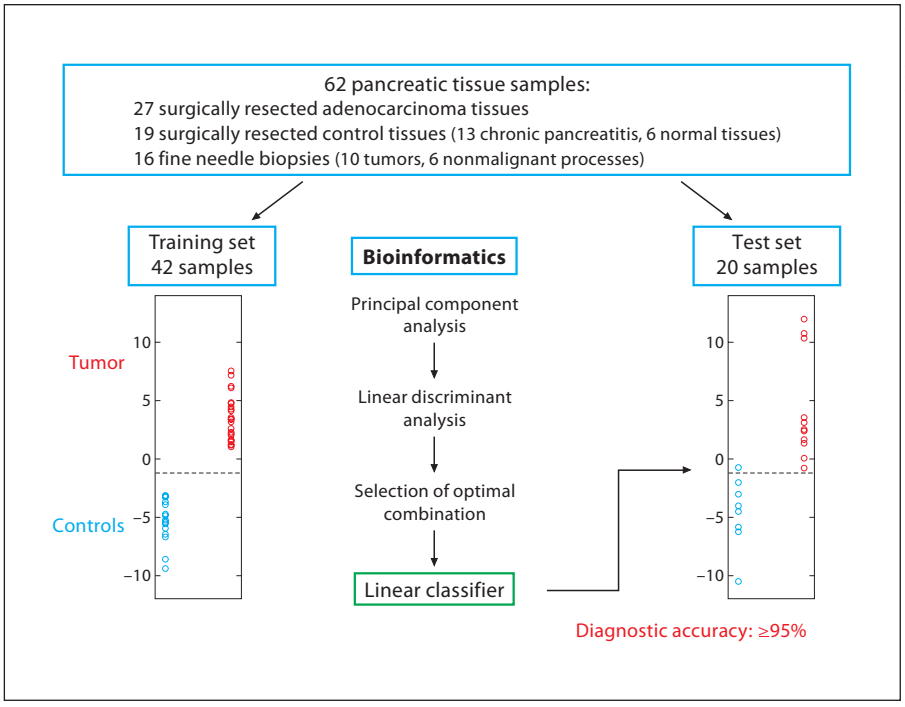

Fig. 2. Flow chart outlining the process of construction and evaluation of a classification algorithm to differentiate PDAC from benign samples. 62 pancreatic tissue samples, including 16 FNAB samples, were divided into a 42-sample training set and an independent 20-sample test set. In the first step, principal component analysis was performed on the training dataset to reduce the dimensionality of the data. Subsequently, linear discriminant analysis was performed on the first 30 principal components of the dataset. The combination of principal components resulting in the greatest separation between tumor and control samples was selected to define the linear classifier (left panel: tumor and control samples were plotted according to their relative distances to the separating hyperplane). The classifier was then evaluated using the independent 20-sample test set, demonstrating ≥95% diagnostic accuracy (right panel: one benign control sample is falsely classified as PDAC). Modified from Buchholz et al. [86].

as a sensitive, specific and early marker for pancreatic cancer in biopsy material.

A considerable number of studies has investigated the use of Mucin genes and proteins for the differential diagnosis of pancreatic cancer. Mucins are high molecular weight glycoproteins which are expressed in a variety of epithelial cell types. Overexpression or de novo expression in pancreatic cancer has been reported for different mucins [73–76], and detection of mucin mRNA or protein in pancreatic juice [77], FNABs [78–81] or tissue samples [75, 82] for diagnostic purposes has been explored by different groups. However, the reported results vary considerably and are often contradictory, so that a clear picture is not yet emerging.

An alternative to the analysis of changes in the abundance of transcripts or proteins is the detection of aberrant DNA methylation. Promoter CpG island methylation is a common gene-silencing mechanism in cancerogenesis [83] and is readily detectable using methylation-specific PCR. Matsubayashi et al. [84] demonstrated that quantitative analysis of the methylation status of the genes *Cyclin D2, FOXE1, NPTX2, ppENK, p16*, and *TFPI2* in pancreatic juice was suitable to differentiate between malignant and benign processes of the pancreas with 82% sensitivity and 100% specificity. Moreover, in a recent analysis of 55 ERCP-guided pancreatic duct brush cytology samples, methylation status of the *NPTX2* gene promoter alone predicted malignant disease with 87% sensitivity and 80% specificity [85].

Multiclass Diagnosis

As mentioned in the introduction, rarer malignancies of the pancreas, such as ampullary cancers, acinar cell tumors or neuroendocrine tumors, as well as tumors originating from duodenal tissue or the bile duct which may be mistaken for pancreatic tumors, generally have a better prognosis than PDAC and may require different treatment regimens. While several of the abovementioned single candidate markers or combinations of limited numbers of markers show great potential in the differentiation between malignant and benign processes in the pancreas, none are suitable to differentiate between different entities of pancreatic tumors. Accurate classification of tumor types and subtypes requires a much higher degree of multiplexing, interrogating many markers in parallel. Among the different high-content screening technologies which could provide suitable platforms for the development of such highly multiplexed diagnostic tests, mRNA profiling using DNA microarrays is the most advanced and most readily available technology to

date. Indeed, a multitude of microarray profiling studies which have been conducted in recent years have already accumulated a wealth of information on gene expression profiles of different tumor entities (see above) and have demonstrated that different tumors can be identified by distinct gene expression signatures. However, the use of large scale ('whole genome') arrays is extremely costly and generates vast amounts of data which are difficult to analyze in a routine diagnostic setting. In order to circumvent both of these problems, we have previously designed a specialized 588-feature cDNA array for pancreatic cancer differential diagnosis. In a proof-of-principle study with 62 pancreatic tissue samples, including 16 FNAB samples, we demonstrated that a 169-gene expression signature obtained with this array in conjunction with a specifically developed classification algorithm is suitable to distinguish between PDAC and nonmalignant processes with >95% diagnostic accuracy [86] (fig. 2).

Within the context of the MolDiag-Paca project, we are currently in the process of extending this study to the analysis of additional tumor types in order to develop a multiclass classification system for the comprehensive diagnosis of different malignancies in the pancreas. Preliminary results show that ampullary cancers, which can be very difficult to differentiate from PDAC with conventional diagnostic procedures, have a very distinct diagnostic array expression signature and can readily be distinguished from both PDAC and benign pancreas samples. For these preliminary multiclass classification analyses, a previously published algorithm based on 'shrunken centroids' of gene expression [87] was used. The results showed that while the separation of ampullary cancer samples from the other diagnostic entities was very good, the PAM classifier performed significantly worse than our previously developed algorithm in the differentiation between PDAC and chronic pancreatitis samples [unpubl. data], probably due to the different feature reduction schemes [88]. In addition to the ongoing analysis of additional relevant tumor entities, we are therefore continuing to develop novel algorithms specifically geared towards the evaluation of the diagnostic cDNA array expression profiles.

Summary and Outlook

Currently available routine procedures are inadequate to provide for timely and accurate differential diagnosis of pancreatic tumors. The advent of high-throughput genomic, transcriptomic and proteomic techniques has led to the identification of a large number of molecular differences between malignant and benign processes in the pancreas, and even more importantly between different entities of pancreatic malignancies. Several candidate molecular markers have shown great diagnostic potential in exploratory studies, although these need to be validated in large, controlled, prospective studies. Most single molecular markers serve as relatively unspecific 'indicators of malignancy', providing a general differentiation between malignant and benign processes. However, more differentiated diagnoses such as the distinction of various tumor types or the provision of prognostic information for each individual patient will require the development of multiplexed assays, such as the diagnostic array described above. Ultimately, the ideal molecular diagnostic test may require combinations of tests working at different levels of genome, transcriptome or proteome analysis. The MolDiag-Paca consortium is heavily involved in the identification and evaluation of novel single or multiplex molecular markers on all levels, from the assembly and mining of comprehensive datasets to identify new, promising candidates to the clinical evaluation of previously developed approaches.

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