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## **Fine-Needle Aspirates v2.0 — The Molecular Era**

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Louis Buscail and Pierre Cordelier

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### **Abstract**

Tissue biopsies are required for diagnosis, prognosis, and to measure individual drug response markers for patient management. For pancreatic adenocarcinoma, surgically harvested tissues are often used to collect data and perform genomic analysis to identify driver oncogenes and specific mutations, or to quantify a handpick of (micro)RNAs and proteins biomarkers. However, such strategy raises many concerns not only because 80% of patients diagnosed with pancreatic adenocarcinoma are not eligible for surgery, meaning that biopsies are not collected, but also because repeated core biopsies are related to higher risk of morbidity, are expensive and logistics can be limiting. Alternative sample collection methods include fine-needle aspirates (FNA) collected under endoscopic ultrasound (EUS). In this chapter, we will describe how EUS-FNA material can be a wealthy source of biomarkers for pancreatic cancer patient management. In greater details, we will review how DNA, micro(RNA), or protein analysis can help stratify pancreatic adenocarcinoma patients, from single events analysis, to cutting-edge, high-throughput studies.

**Keywords:** FNA, microbiopsies, pancreatic diseases, NGS, miRNA, digital PCR

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### **1. Introduction**

Tissue biopsies are required for diagnosis, prognosis, and to measure individual drug response markers for patient management. For pancreatic adenocarcinoma, surgically harvested tissues are often used to collect data and perform genomic analysis to identify driver oncogenes and specific mutations, or to quantify a handpick of (micro)RNAs and proteins biomarkers. However, such strategy raises many concerns not only because 80% of patients diagnosed with

pancreatic adenocarcinoma are not eligible for surgery, meaning that biopsies are not collected, but also because repeated core biopsies are related to higher risk of morbidity, are expensive and logistics can be limiting. Alternative sample collection methods include fine-needle aspirates (FNA) collected under endoscopic ultrasound (EUS). In this chapter, we will describe how EUS-FNA material can be a wealthy source of biomarkers for pancreatic cancer patient management. In greater details, we will review how DNA, micro(RNA), or protein analysis can help stratify pancreatic adenocarcinoma patients, from single events analysis, to cutting-edge, high-throughput studies.

## 2. Fine-needle aspirates for pancreatic cancer diagnosis and prognosis

Pancreatic cancer remains one of the most deadly types of tumor. The five-year survival rate after diagnosis is less than 3.5% [1]. Only 15% of pancreatic ductal adenocarcinoma (pancreatic cancer) patients can be diagnosed at a resectable and possible curative stage. The remaining patients diagnosed with locally advanced and/or metastatic tumors are treated in a palliative way. Single-agent gemcitabine, although not dramatically improving survival, has demonstrated a significant clinical benefit and has become the standard chemotherapy for advanced pancreatic cancer [2]. Recently, FOLFIRINOX protocol and association gemcitabine-Nab-paclitaxel regimens were found to improve the survival of metastatic patients when compared to gemcitabine alone [3, 4]. However, median survival does not exceed six and ten months for metastatic and locally advanced pancreatic cancer, respectively [5]. One way to improve pancreatic cancer management is to establish a rapid and clear diagnosis in order to operate or to start a medical treatment as soon as possible. One of the critical clinical conditions is the differentiation of pancreatic cancer from focal pancreatitis. It is indeed necessary to avoid unnecessary resection of benign lesions (such chronic pancreatitis in its pseudotumoral form or autoimmune pancreatitis) or to delay the treatment of pancreatic cancer in a subset of patients. Recent advances in abdominal imaging techniques may favor a more rapid histological diagnosis and also may resolve some of these problems of differential diagnosis. Among these imaging techniques, endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is a rapid, safe, cost-effective, and accurate technique for evaluating and staging pancreatic tumors [6–8]. EUS allows to guide FNA of solid pancreatic lesions for cytopathological analysis. However, its performance for the diagnosis of malignancy varies widely with a sensitivity ranging from 75–94% and an accuracy of 78–95%. In addition, if the specificity approaches 100%, its negative predictive value ranges from 40–75%. In addition, EUS-FNA may be inconclusive in up to 20% of cases [8, 9]. Overall, false-negative rates and atypical or suspicious diagnoses remain relatively frequent using cytopathological analysis [9]. The improvement of molecular biology techniques, including DNA and RNA amplification, permits the analysis and quantification of molecular markers in cytological samples. In addition, EUS-FNA is the main clinical appliance for cytological and histological material collection from locally advanced pancreatic cancer that represents 85% of pancreatic cancer patients. This chapter depicts the widespread potential for the molecular analysis of samples obtained by EUS-FNA in conducting translational studies to improve knowledge and diagnosis of pancreatic cancer.

### 3. Identification of key mutations in tumor suppressor genes and oncogenes using FNA material

We and others have demonstrated that DNA extracted from EUS-FNA material may permit to detect single DNA mutations in key tumor suppressor genes and oncogenes for better pancreatic cancer patients management (for review [6]). The next challenge is to identify new technologies that are more sensitive than standard quantitative PCR (qPCR) or that may allow screening of multiple mutations in a few nanograms of DNA. Recently, digital PCR (dPCR) has shown promise in cancer diagnosis, although current dPCR systems have lower throughput than qPCR systems. dPCR is based on absolute nucleic acid quantification following partitioning of individual molecules into multiple replicate reactions at limiting dilution. Following reaction, the starting concentration of template is determined by Poisson statistical analysis of the number of positive (with amplified target) and negative (without amplified target) detected reactions [10]. By essence, dPCR is anticipated to be more tolerant to PCR inhibitors by virtue of being an end-point approach; with improved amplification efficiency and, thus, sensitivity. Accordingly, Hindson et al. recently compared the performance of dPCR to real-time PCR in clinical specimens. When applied to serum microRNA biomarker analysis, dPCR was proved to provide superior diagnostic performance for identifying individuals with cancer than qPCR [10]. Similar results have been reported in a small cohort of patients with pancreatic cancer. Dr Capella's group recently demonstrated that digital PCR provides a robust and quantitative assay for KRAS mutant alleles detection in routinely obtained samples [11]. The next objective is to transfer this approach to the screening of DNA mutations in material from EUS-FNA.

Large pancreatic cancer sequencing initiatives are revealing a vast array of molecular aberrations in histologically indistinguishable tumors (for review [12]). Of importance, the mutational burden seems to be particularly heterogeneous and this has major implications in therapeutic development and clinical care. Indeed, better understanding the genetic and molecular basis of cancer may not only help to stratify patients but also develop new classes of therapies that selectively target molecular mechanisms that are essential for the survival and proliferation of cancer cells. Unfortunately, such analysis requires amounts of material that are out of reach following EUS-FNA. Alternatively, targeted next-generation sequencing (NGS) can be performed with minimal amount of DNA. One of these approaches is based on the Ion Torrent Ampliseq technology. In thyroid cancer, 5–10 ng of input DNA is sufficient for the successful analysis of virtually all samples, either thyroid tissue or fine-needle aspiration samples, and revealed point mutations in specific types of thyroid cancer [13]. We recently translated this method to the analysis of FNA material from pancreatic tumors; we found that this approach is feasible with minimal amount of DNA. Using the comprehensive cancer panel, we verified KRAS mutation and discovered new mutations in key oncogenic drivers and tumor suppressor genes that are currently validated by qPCR. Thus, targeted NGS holds great promise for pancreatic cancer patient stratification and management.

#### 4. Quantification of microRNA in FNA material

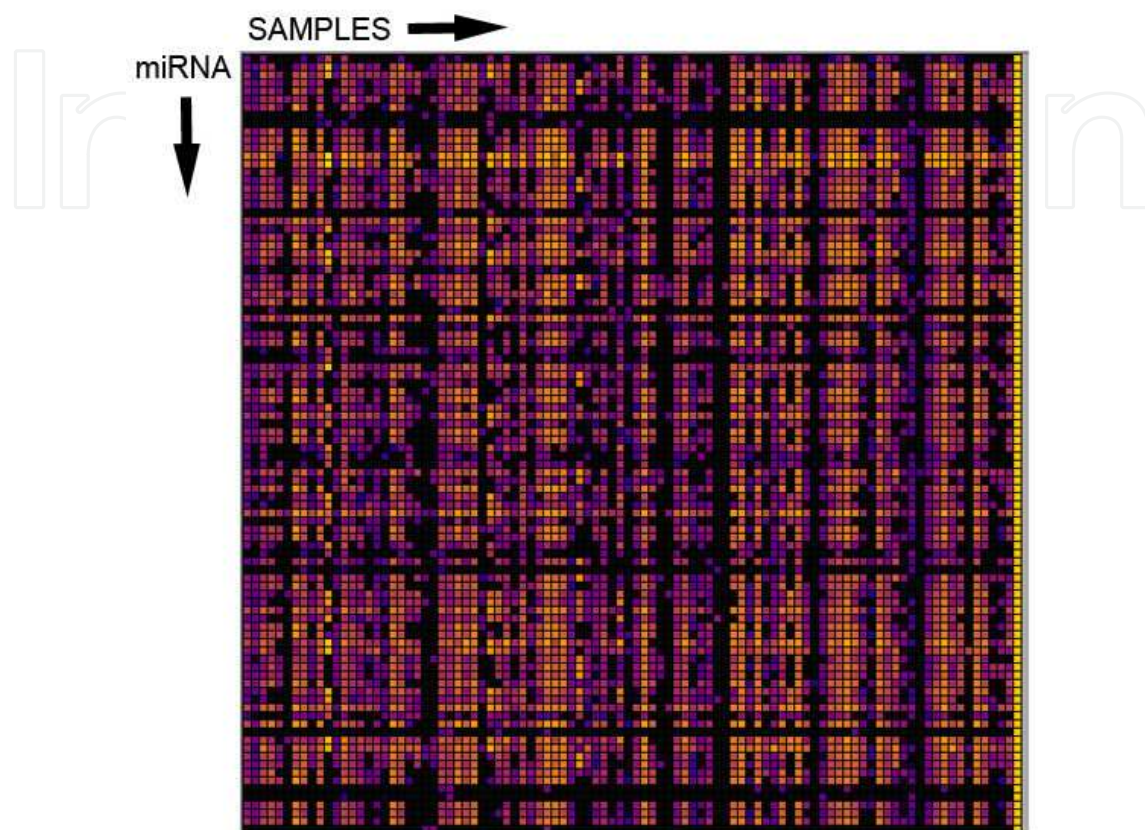
Beside DNA, microRNAs are well-characterized biomarkers for pancreatic cancer management (for review [14]). To date, most studies are limited to the quantification of a handful of selected candidate microRNAs in EUS-FNA material. For example, Dr Szafranska's group recently validated a 5-miRNA expression classifier to improve preoperative detection of pancreatic cancer [15]. While these results are encouraging, further developments are needed to analyze simultaneously several dozens of microRNA in EUS-FNA material. Current methods for the detection and measurement of microRNA expression include the abovementioned standard quantitative PCR and microarray based analysis. However, these methods suffer several limitations when used in large clinical studies where a high-throughput and highly quantitative technology is needed for the efficient characterization of a large number of microRNA transcripts in clinical samples. Recently, high-throughput qPCR-based microfluidic technology (Biomark Fluidigm) has been evaluated to quantify microRNA expression in lung cancer [16]. The authors demonstrate that (1) the technic is highly reproducible, (2) multiplex results correlate closely with singleplex qPCR, (3) throughput is 5–20 times higher, and (4) cost is approximately 50–100 times lower than conventional assays [16]. We recently benchmarked and validated this approach for the analysis of 96 candidate microRNAs in EUS-FNA material from pancreatic cancer patients, starting from 200 ng of total RNA (Fig. 1). In addition, we found that amplification quality was greater when using material collected from FNA and conserved in RNA later, as compared to material extracted from archived slides (personal observation).

While the measurement of microRNA expression in rare cell populations or in precious samples such as FNA still poses practical challenges due to the low amount of RNA present, alternative techniques have been developed to quantify the complete microRNA landscape in less than 50 ng of total RNA. Such highly sensitive real-time quantitative PCR strategy utilizes microfluidic array cards containing prespotted TaqMan probes that allows the detection of mature miRNAs in small reaction volumes. This approach, namely OpenArray® MicroRNA Panels, was used for the characterization of microRNA expression in mouse hematopoietic stem cells [17]. We have recently validated this approach for the detection and quantification of circulating microRNA in patients (Buscail *et al.*, Molecular Therapy, in press); similar experiments are currently ongoing to characterize the microRNA pattern of expression in EUS-FNA material from patients with pancreatic cancer.

#### 5. Multiplexed protein analysis in FNA material

EUS-FNA has been proven as a useful method for diagnosing pancreatic lesions and is associated with high accuracy in moderately to poorly differentiated pancreatic cancer. However, diagnosis of well-differentiated cancer or early stage tumors can be challenging; in these situations, an indeterminate diagnosis is often rendered. Ancillary stains can provide much needed supportive evidence. Many studies have used a large number of stains to





**Figure 1.** qPCR-based microfluidic analysis of microRNA expression in pancreatic tumors FNA. *Unpublished results.*

document an immunohistochemical profile of pancreatic cancer [18–25], but the number of proteins than can be analyzed is often limiting. Recently, multiplexed flow cytometry and mass cytometry have been proposed to examine an expanded set of markers (up to 50). However, flow cytometry is often limited by the number of markers that can be analyzed due to spectral overlap. Also, mass cytometry requires cells to be vaporized during sample preparation, resulting in sample loss [26]. Very recently, Ullal *et al.* designed a remarkable strategy based on antibody barcoding with photocleavable DNA platform to perform multiplexed protein measurements in small amounts of clinical sample material [27]. This method showed high reproducibility and achieved single-cell sensitivity. Using this strategy, the authors successfully analyzed the expression of 90 candidate proteins and demonstrated that this method could be used to identify pathway responses to molecularly targeted drugs in FNA, to help predict drug response in patients with lung cancer [27]. While technically challenging, it is tempting to speculate that this approach will be soon translated to the analysis of EUS-FNA material from patients with pancreatic cancer.

## 6. Future development: Defining pancreatic tumors heterogeneity at the cellular level

As stated before, large-scale genomic studies on pancreatic tumors revealed marked inter- and intratumoral heterogeneity and complexity, and may explain the lack of success of conventional, disease-based approaches, therapies. A better understanding of the underlying molecular pathology at the cellular level may undoubtedly lead to novel therapeutics development. Indeed, many biological programs are performed under the assumption that all cells of a particular type are identical. However, recent data suggest that individual cells within a single population may differ quite significantly and these differences can drive the health and function of the entire cell population, including major variation in the tumor cell microenvironment. Single cell analysis comprises a broad field that covers advanced optical, electrochemical, mass spectrometry instrumentation, and sensor technology, as well as separation and sequencing techniques. Although the approaches currently in use can offer snapshots of single cells, the methods are often not amenable to longitudinal studies that monitor changes in individual cells *in situ*. Recently, David Ting *et al.*, from Dr Harber's group in Harvard, have performed epitope-independent microfluidic capture, followed by single-cell RNA sequencing, to analyze circulating pancreatic tumor cells (CTCs) in experiment models [28]. The authors demonstrate that CTCs exhibit a very high expression of stromal-derived extracellular matrix proteins, including SPARC, a tentative prognosis marker for nab-paclitaxel-based therapy. At present, the clinical use of single-cell analysis is – with the exception of preimplantation diagnosis – still in its infancy. However, we are facing an era of integrated single-cell genomic, epigenomic, transcriptomic, and proteomic analysis that will revolutionize whole-organism science. Single-cell diagnostics will be instrumental for the realization of personalized medicine for pancreatic cancer patients and for the development of completely novel therapeutic concepts.

## 7. Conclusion

At present, the classification of tumors is mainly based on observational characteristics, such as morphology, phenotype, or developmental origin. The current progress in developing sophisticated approaches for investigating EUS-FNA material will definitely improve our understanding of pancreatic cancer at multiple levels, through (1) a better definition of cell types and intercellular variability, (2) a possibility to carry large-scale mutational, transcriptomic, or epigenomic analyses, and (3) an improved identification of rare cell types, to play an increasing role in the detection of minimal residual disease or in the analysis of circulating tumor cells. Accordingly, there are existing opportunities for more rapid improvement in outcomes by adopting a more stratified or personalized approach using markers from different molecular species. However, these progresses will have to complain not only with the development of new drugs but also with clinical care and regulatory agencies. Nonetheless, it is tempting to speculate that EUS-FNA-based molecular evidences will soon drive decision making for patients with advanced pancreatic cancer.

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