Liquid Biopsy in Pancreatic Cancer

Abstract
Pancreatic cancer is the 9th most frequent cancer but it stood as the 4th cause of cancer death in 2016 and it is one of the few cancers in which mortality keeps increasing. Due to the inherent difficulty of this cancer for an early diagnosis and its relatively difficult access for performing a biopsy, increasing efforts are being done for developing liquid biopsy in PDA. While our knowledge of the biology of PDA has notably improved in recent years, the development of modern targeted therapies needs to be coupled to the existence of reliable biomarkers for monitoring. Different tumor constituents such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and extracellular vesicles (EXV), are shed into the circulation by tumors and can be detected in the peripheral blood of patients with cancer. This review will summarize the advances that have recently been made in the field of liquid biopsy for pancreatic cancer.

Keywords: Pancreatic cancer, Pancreas, Liquid Biopsy, Circulating tumor cells, Circulating tumor DNA, Extracellular vesicles, Exosomes, CTC, ctDNA, Digital PCR, Peripheral blood

Abbreviations: PDA: Pancreatic Ductal Adenocarcinoma; CTCs: Circulating Tumor Cells; ctDNA: Circulating Tumor DNA; EXV: Extracellular Vesicles; NGS: Next Generation Sequencing; OS: Overall Survival; EpCAM: Epithelial-Cell Adhesion Molecule; NE-iFISH: Negative Enrichment Method Coupled to Immunofluorescence in Situ Hybridization; ddPCR: Digital Droplet PCR; TEX: Tumor Exosomes; MAF: Mutant Allele Frequency

Introduction
Pancreatic ductal adenocarcinoma (PDA) constitutes one of the main causes of cancer death in the external World. In the USA it was estimated that in 2016 it was the 9th most frequent cancer. Despite its moderate incidence, it stood as the 4th cause of cancer death and it is expected to be the second deadliest cancer by 2030 [1]. This discouraging data are explained, at least in part, by the difficulty for an early diagnosis due to the absence of symptoms in early stages and low yield of imaging tests, and by the poor therapeutic results in more advanced stages. Indeed, median survival in patients with metastatic disease is less than a year with current chemotherapy regimens [2,3]. While our knowledge of the biology of PDA has notably improved in recent years, the development of effective therapies is hampered by the relative inaccessibility of this tumor to traditional biopsy. Therefore, increasing efforts are being done to refine liquid biopsy technologies in PDA. Different tumor constituents such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and extracellular vesicles (EXV) can be detected in the peripheral blood of patients with cancer, and also in patients with PDA. Most studies of liquid biopsy have focused in applying it as a prognostic biomarker or as a tool for monitoring response to treatment. This review will summarize the most relevant advances in liquid biopsy for patients with pancreatic cancer.

Circulating tumor cells
Several research groups have provided interesting data in the past few years in the field of CTCs in pancreatic cancer. However, CTC technologies are hampered by a still low-to-moderate sensitivity of detection. While various studies have shown the prognostic value of CTCs in PDA, the still low isolation capacity of most CTC technologies, limits the amount of DNA and RNA that can be obtained for conducting molecular studies, and therefore their value as a predictive tool is debated [4,5]. Nevertheless, the advent of digital PCR and Next Generation Sequencing (NGS) have improved the quality of molecular studies performed on CTCs, since they need very small amounts of sample [5]. Kullemann et al. [6], using a size-based detection method, found CTCs in 8 of 10 patients with localized PDA and in 10 out of 11 patients with locally advanced or metastatic disease. They studied the KRAS pG12V mutation in CTCs, and found it predicted a longer overall survival (OS) (19.4 vs 7.4 months, P=0.015). Earl et al [7], using CellSearch®, an EpCAM-based isolation method, and detected CTCs in 7 out of 35 patients (1 patient with localized disease and 6 with metastases). In this study the presence of CTCs associated with a worse prognosis (OS CTC+ vs CTC: 88 vs 395 days, P=0.0108). Sergeant et al. [8], using a method based on negative depletion of CD45+ and CD34+ cells, detected the presence of CTCs in 6/10 patients after tumor resection. The authors demonstrated the expression in CTCs of a gene signature associated with cell migration. Khoja et al. [9], compared the CellSearch® and ISET methods-the latter a size-based detection method- in a prospective set of 54 patients with PDA in different stages. ISET detected CTCs in 93% of the patients comparing favorably with the 40% detection rate of CellSearch®, and the median number of CTCs was also higher with ISET. Bidard and colleagues [10], using the CellSearch® method, found CTCs in 5% and 9% of 79 patients with locally-advanced PDA before, and...
after 2 months of chemotherapy, respectively, and their presence
associated with a worse prognosis.

Dotan et al. [11], used CellSearch® in a prospective series of
48 patients with metastatic PDA, and detected CTCs in 48% of
them. They observed a worse prognosis in patients who’s CTCs
expressed MUC-1, a transmembrane glycoprotein related to
epithelial to mesenchymal transition. Finally, Xu et al. [12], used
a negative enrichment method coupled to immunofluorescence
in situ hybridization (NE-iFISH) of chromosome 8 to detect CTCs.
This method was able to detect CTCs in 90% of 40 patients in
different disease stages, and they established ≥ 3 triploid CTCs
as the cut-off predicting a worse 1-year OS (P=0.0279) and OS
(P=0.0188). They also showed that the presence of circulating
tumor microembolus associated with a poor response to
chemotherapy in stage IV disease.

The presence of CTC is generally associated with a worse
prognosis, although there is no established cut-off with
prognostic value in PDA. While CTC recovery improves at higher
disease stages, specificity remains high with most CTC detection
systems in all stages. To date, size-based and negative enrichment
immunoaffinity methods have shown a greater isolation
efficiency, with sensitivities ranging from 80-90% compared to
5-48% of standard EpCAM-based technologies. The former could,
therefore, be useful for improving early diagnosis of PDA.

**Circulating tumor DNA**

Similar to CTCs, although to a lesser extent, the detection of
tDNA is hampered by assay sensitivity. Indeed, most solid results
have appeared in the past few years with the use of digital PCR
and NGS methods. The group of Däbritz et al. [13], detected
KRAS mutations in 36% of 56 patients with PDA using real-time
PCR. Using direct sequencing, Chen et al. [14], detected KRAS
mutations in 33% of 91 patients, and they predicted a worse
outcome in patients with unresectable disease. Bettegowda et al.
[15], used digital PCR in a large sample of 155 patients with PDA,
detecting tDNA in more than 75% of patients with advanced
disease and in as much as 57% of patients with localized disease.
The group by Earl et al. [7], using digital PCR, detected tDNA in
31 out of 35 patients with PDA, and found a mutation in KRAS
in 8 of them, associated with a worse prognosis (OS KRAS
mutant vs KRAS wild-type: 66 vs 772 days, P=0.001). Takai et
al. [16], in another large sample of 95 patients with PDA stages
I-III and in 163 patients with stage IV disease, using digital
droplet PCR (ddPCR) and targeted deep sequencing, identified
potentially targetable mutations in tDNA in 29.2% of the cases.
Sausen et al. [17], by means of ddPCR, identified tDNA in 43% of
51 resected stage II PDAs. Besides, the presence of tDNA
after tumor resection predicted recurrence 6 months in advance
of clinical recurrence. Finally, in a large series by Kinugasa et al.
[18], mutation in KRAS in tDNA was detected in up to 62.6%
of patients by ddPCR with a concordance of 77.3% with KRAS
mutation status in solid tumor. While the mutation in KRAS
in the primary tumor had not prognostic value, the presence
of mutant KRAS in tDNA was associated with a significantly shorter
survival (P=0.006). Studies using digital PCR have demonstrated
very high sensitivities, either in localized stages of PDA (up to 57%
sensitivity) or in advanced stages (up to 88% sensitivity), for the
detection of frequent mutations in DNA hot-spots such as those in
KRAS. Indeed, KRAS-mutant tDNA in PDA has been recurrently
associated with a worse prognosis. Due to the high prevalence
of KRAS mutations in PDA, it may also serve in early PDA detection
and in therapy monitoring [19].

**Extracellular vesicles**

ExV are membrane-bound vesicular components shed by cells
into the peripheral circulation and other body fluids. They differ
in size and molecular cargo, and can be classified as apoptotic bodies
(1000-5000 nm), intermediate-sized microvesicles (200-1000
nm), and exosomes (30-150 nm) [20]. The latter are a rich source
of proteins, mRNA and DNA. Indeed, they have been recently
recognized as the most important reservoir of tDNA in plasma
[21,22]. Besides, mutations in DNA from tumor exosomes (TEX)
consistently reflect the mutational status of their parental cells
[20,21,23]. TEX has been studied in many solid tumors, including
PDA. They can be easily obtained by differential ultracentrifugation
and are identified by the expression of some membrane proteins
known as tetraspanins (CD63, CD9, CD81) [20]. Recently, Melo
et al. [24], identified glipican-1, as a highly-sensitive and specific
marker of TEX derived from patients with PDA (100% sensitivity
and specificity), allowing to differentiate patients with PDA and
with premalignant lesions from patients with benign pancreatic
disease and healthy controls. As mentioned before, TEX is also
shed to other body fluids, such as saliva. In a murine model of PDA,
the presence of TEX was indirectly demonstrated in the saliva
and they have already been detected in saliva from patients with
lung cancer [25]. Due to the advantages of studying TEX (easy
acquisition, potential for molecular studies, moderate costs), there
is a growing interest in TEX as a liquid biopsy tool for early cancer
detection and treatment monitoring, particularly in PDA. Indeed,
Alleson et al. [26] have recently shown results of a large study
comparing KRAS mutation detection by digital PCR in tDNA and
DNA obtained from TEX in patients with localized and advanced
PDA. They found a sensitivity and specificity of 75.4% and 92.6%
respectively, for the detection of mutant KRAS in TEX DNA. They
also detected a higher percentage of mutant KRAS in TEX DNA
from patients with early stage disease (43.6%) than previously
reported and observed that a higher mutant allele frequency
(MAF) of KRAS in TEX DNA was associated with a poor prognosis
in these patients. However, up to 20% of healthy controls showed
mutant KRAS in TEX DNA, demonstrating the need for improving
the specificity of exosome isolation. As mentioned earlier, the
discovery of glipican-1 as a TEX-specific biomarker for PDA, may
help to refine their role as a liquid biopsy tool [24].

Although to date only a few studies have been reported using
TEX in PDA, are a promising tool for liquid biopsy due to their
easy acquisition with very high sensitivities (75-100%) and
specificities (92-100%). They seem especially valuable in early
cancer detection and for therapy monitoring due to their rich
molecular cargo. However, their prognostic value has yet to be
established.
Conclusion

Several tumor components such as circulating tumor cells, circulating tumor DNA and exosomes are shed into the peripheral circulation and can be detected in patients with pancreatic cancer. Although each detection technology has different applications, all of them may have great utility in pancreatic cancer diagnosis and in monitoring therapy. In the near future, liquid biopsy will certainly become a reality in the daily clinical practice of pancreatic cancer.

References
