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Characterization of somatic mutations profile in pancreatic adenocarcinoma

PhD THESIS SUMMARY

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Fundamental problem

Pancreatic adenocarcinoma has an increasing incidence and a dismal prognosis (1). It is currently the seventh leading cause of cancer related death globally, but is projected to become the third leading cause of cancer death in the United States by 2040 (2). This phenomenon is explained by the increase in the prevalence of its risk factors – obesity, diabetes and also the increase in life expectancy (3). In addition, the prognosis of pancreatic adenocarcinoma is dismal, the 5-year survival does not exceed an average of 9% for all disease stage, and has not seen remarkable improvement in recent decades despite major advances in oncology (1). Among the causes of this increased mortality of pancreatic cancer are, on the one hand, the diagnosis of the disease in advanced stages ineligible for therapies with a curative visa, and on the other hand, an increased resistance to chemo-radiotherapy (4). The diagnosis is established in advanced stages because, in early stages, the disease is asymptomatic, or causes non-specific symptoms easily attributed to other benign pathologies (4). Also, since the incidence in the general population is low compared to the incidence of colorectal or breast cancer, the application of screening methods is unfeasible – even a test with sensitivity and specificity of 99% would produce a large number of false negative results, which would generate anxiety for the subjects in this category and important healtthcare costs without real benefits (4). The current approach in this regard is to follow with imaging methods subjects known to have a significantly increased risk of pancreatic cancer compared to the general population - such as those with familial pancreatic cancer, various genetic syndromes, patients with newly diagnosed diabetes or patients with conditions with the potential for progression to pancreatic adenocarcinoma such as those with chronic pancreatitis or pancreatic mucinous cysts (3,4). Regarding resistance to treatment, it is considered that, in addition to the intensely desmoplastic stroma that characterizes pancreatic adenocarcinoma and that does not allow the distribution of systemic treatments at the tumor level, multiple molecular alterations occur in the process of carcinogenesis that allow tumor development to continue under antineoplastic treatment (1,4). All these elements make pancreatic adenocarcinoma the most redoutable solid tumor for both the patient and the management team.

Remarkable advances in the field of genetics and biomedical engineering have led to the discovery of subtle pathophysiological mechanisms, especially in neoplastic pathologies, and

to a paradigm shift with a major impact in oncology and beyond - namely personalized therapy - based on the tumor molecular profile. The effect of this phenomenon in pancreatic cancer, however, is still limited. The genetic mutations that occur in approximately 90% of pancreatic adenocarcinomas, from the early stage of the disease, are mutations of the KRAS gene, which until recently was considered inaccessible by targeted treatment (5). The potentially actionable molecular alterations that appear in pancreatic cancer have a low individual incidence, but cumulatively, they can be detected in a quarter of patients with pancreatic cancer (5). There are currently molecules that target KRAS12C mutations that have also proven effective in pancreatic cancer, but KRAS12C mutations rarely occur in this tumor type, and targeted therapies are being developed for the more common KRAS mutations in pancreatic cancer such as KRAS12D (6)(7).

Knowing the mutational profile of pancreatic cancer has become an indispensable premise for a treatment that offers maximum benefits to the patient. Genetic testing is currently routinely recommended for first-degree relatives of patients with pancreatic adenocarcinoma, to assess its familial aggregation, and also for patients with advanced disease to guide targeted therapies (3). In addition, sustained research efforts address the possibility of using genetic testing both for early disease diagnosis and to guide its management (8).

In the present work, we approach two main directions regarding the mutational profile in pancreatic adenocarcinoma - on the one hand - the possibility of using tumor samples obtained by fine needle aspiration under echoendoscopic guidance, for next generation generation sequencing and on the other hand the possibility to use liquid biopsy methods to evaluate its prognosis.

Hypothesis and general objectives

The roles of ultrasound-guided tissue aquistion in pancreatic cancer have expanded, from tumor diagnosis to obtaining adequate samples for molecular testing (9–11). Although, according to current guidelines, tissue fragments should be used for molecular testing in solid pancreatic lesions, there is increasing evidence for the feasibility of performing next-generation genetic testing based on FNA samples

Free circulating DNA and circulating tumor DNA (ctDNA) represent the most studied type of liquid biopsy in PDAC (12). Circulating cell-free DNA consists of short chains of nucleic acids released by cells, mainly during necrosis or apoptosis, and which carry genetic and epigenetic modifications specific to the cell of origin (13). There is increasing evidence regarding the association of cfDNA biomarkers with tumor stage, tumor burden, and prognosis in PDAC (14–17). There is preliminary evidence suggesting that ctDNA detection is associated with the presence of micrometastases and could predict tumor recurrence before it becomes detectable by imaging methods, thus showing great potential for disease monitoring (18,19).

Exosomes are extracellular vesicles of nanometric size (30-150 nm), of endosomal origin, released physiologically (20,21). They can act on the target cells either through the endocrine pathway being released into the systemic circulation for this purpose, or through paracrine and autocrine mechanisms by activating specific signaling pathways (20,21). They mainly transport nucleic acids, lipids and proteins protected from degradation in the extracellular environment by a lipid bilayer (20,22). In pancreatic cancer, exosomes are involved in processes such as epithelial-mesenchymal transition, cell proliferation, angiogenesis, premetastatic niche formation, thus favoring tumor development and spread (22,23). Although the isolation of exosomes is laborious, they can accurately reflect tumor heterogeneity through the variety of their molecular content having stability in the extracellular environment (24,25).

The working hypothesis of this thesis is that the results of molecular tests in pancreatic adenocarcinoma using tumor samples aquired by echoendoscopy and liquid biopsy methods are complementary and indispensable for guiding personalized management in pancreatic adenocarcinoma that can improve its prognosis

The general objectives include, in this sense: the evaluation of the possibility of using fine needle aspiration samples for the characterization of the profile of somatic mutations in

pancreatic adenocarcinoma by evaluating the parameters of DNA extracted from this type of samples and their use for next-generation sequencing,- and this was performed through a cohort study. Another general objective was to evaluate the possibility of using liquid biopsy methods to guide the management of pancreatic adenocarcinoma by characterizing their prognostic role - specifically of circulating cell-free DNA and exosomal biomarkers - for this purpose, two systematic reviews and meta- analyzes for each type of biomarkers were performed.

Chapter 1. Somatic mutations profile in pancreatic adenocarcinoma diagnosed by endoscopic ultrasound guided fine needle aspiration

Genetic testing is increasingly being performed for the management of unresectable pancreatic cancer. For this purpose, current guidelines recommend the use of tissue fragments, albeit based on moderate-quality evidence. However, fine needle aspiration (FNA) is most often used in practice among the methods of tumor acquisition under endoscopic ultrasound (EUS) guidance. The aim of this study was to assess the quality of DNA isolated from pancreatic adenocarcinoma (PDAC) samples acquired by EUS-FNA for next-generation sequencing (NGS).

METHODS

We conducted a cohort study. Between November 2018 and December 2021, 105 patients with PDAC confirmed by EUS-FNA were included in the study. Either 22 gauge (G) or 19G needles were used for FNA. One passage of FNA was devoted to subsequent DNA extraction. The concentration (ng/μL) and purity (A260/280, A260/230) of DNA were assessed by spectrophotometry. We assessed differences in DNA parameters according to needle size and tumor characteristics (size, location) and quality of extracted DNA for NGS (adequate material defined as A260/280 ≥ 1.7 and DNA amount: ≥ 10 ng for NGS amplicon-based targeting, ≥ 50 ng for whole exome sequencing [WES], ≥ 100 ng for whole genome sequencing [WGS]). For this purpose we performed the analysis of variance and respectively the t-test for independent observations. We also evaluated the parameters of DNA purity between in relation to the amount of isolated DNA.

RESULTS

Our cohort included 49% men, aged 67.02 ± 8.38 years. 22G needle was used in 71% of cases. DNA parameters in our samples varied as follows: amount of DNA: 1289 ng (interquartile range: 534.75-3101), A260/280 = 1.85 (1.79-1.86), A260/230 = 2.2 (1.72-2.36). We isolated > 10 ng DNA from all samples and > 100 ng in 93% of them (one sample yielded < 50 ng DNA). There were no significant differences in concentration and A260/280 between samples by needle size. Needle size was the only independent predictor of A260/230 that was higher in 22G samples (P = 0.038). The rate of samples suitable for NGS was 90% for 19G

needles, regardless of NGS type, and for 22G needles - 89% for WGS suitability and 91% for WES and amplicon-based NGS, respectively. Samples that yielded >100 ng DNA had significantly higher A260/280 (1.89 ± 0.32 vs 1.34 ± 0.42, P = 0.013). Tumor characteristics were not significantly correlated with DNA parameters.

CONCLUSION

Pancreatic adenocarcinoma samples obtained by fine-needle aspiration under endoscopic guidance yield DNA suitable for NGS. The amount of DNA was similar between 22G and 19G FNA needles. DNA purity parameters can vary indirectly with needle size.

Chapter 2. Prognostic role of cell-free DNA in pancreatic adenicarcinoma – asystematic review and meta-analysis

This systematic review and meta-analysis assessed the prognostic role of circulating free DNA (cfDNA) in pancreatic ductal adenocarcinoma (PDAC). Eligible studies reported differences in overall survival (OS) and progression-free survival (PFS) according to cfDNA parameters. The random-effect model generated hazard ratios (HR) and 95% confidence intervals (CI). Positivity of cfDNA biomarkers was defined as: detection of circulating tumor DNA (ctDNA), detection of KRAS mutations and other cfDNA alterations. In total, 38 studies (3,318 patients) met the eligibility criteria. Progression-free survival (HR = 1.92, 95% CI: (1.29, 2.86)) and overall survival (HR = 2.25, 95% CI: (1.73,2.92)) have were significantly reduced in cases with detectable cfDNA. Similar results were obtained in the case of KRAS mutation detection for both PFS (HR = 1.88, CI: 1.22,2.92) and OS (HR = 1.52, 95% CI:(1.22,1.90)), including all disease stages in the analysis. For non-resectable disease stages, the risk of progression was significantly higher in cases with detectable ctDNA (HR = 2.50, 95% CI:(1.94,3.23)).Results were not similar for unresectable cases with detectable KRAS mutations (HR = 1.16, 95%CI:(0.46, 2.94)). Positivity of cfDNA biomarkers correlated with altered prognosis in both resectable stages of disease and if detected during treatment. In conclusion, cfDNA biomarkers indicate accelerated disease progression and decreased survival in PDAC. Further studies are needed to establishing the significance of detecting KRAS mutations in non-resectable stages of the disease.

Chapter 3. Prognostic role of exosomes in pancreatic adenocarcinoma – a systematic review and meta-analysis

We performed a systematic review and meta-analysis to evaluate the prognostic role of exosomal biomarkers in pancreatic ductal adenocarcinoma (PDAC). The systematic search was performed on January 18, 2021 in MEDLINE, Embase, Scopus, Web of Science, and CENTRAL. Studies reporting differences in overall survival (OS) and progression-free survival (PFS) in PDAC patients according to the status of circulating exosomal biomarkers were included in the review. The random-effect model estimated multivariate (AHR) and univariate (UHR) hazard ratios and corresponding 95% confidence intervals (CI). Eleven studies comprising 634 patients were eligible for meta-analysis. Detection of exosomal biomarkers indicated an increased risk of early mortality (UHR = 2.81, CI: 1.31 6.00, I2 = 88.7%, P < 0.001) and disease progression (UHR = 3.33, CI :2.33 4.77, I2 = 0, P = 0.879) in different stages of PDAC. Detection of exosomal biomarkers preoperatively in resectable stages revealed a higher risk of early mortality (UHR = 5.55, CI: 3.24 9.49, I2 = 0, P = 0.898). No significant differences in overall survival were detected in non-resectable disease stages with positive exosomal biomarkers (UHR = 2.51, CI: 0.55 11.43, I2 = 90.3%, P < 0.001). The detection of certain types of exosomal microRNA was associated with early mortality (UHR = 4.08, CI: 2.16 7.69, I2 = 46.9%, P =0.152) in different disease stages. These results reflect the potential of exosomal biomarkers for prognostic assessment in PDAC. The heterogeneity associated with the results reflects the variability of the methods used in the studies that reported eligible results and the need to standardize them in order to use this type of biomarkers in the clinic.

Conclusions and personal contributions

Personal contributions

Our cohort analysis showed that FNA needle size does not influence the concentration and primary purity parameter (A260/280) of the DNA extracted from pancreatic adenocarcinoma samples when using 19G or 22G EUS FNA needles. However, needle size was the only independent predictor of A260/230. Tumor diameter and location did not influence DNA purity and concentration parameters. Based on the purity thresholds and according to the amount of DNA isolated, there were no significant differences in the sample suitability rates for NGS between the two FNA needle sizes. Sample concordance rate for NGS was 90% for samples obtained with 19G needles regardless of NGS type, while for samples obtained with 22G needles it was 89% for WGS and 91% for both WES , as well as for amplicon-based NGS. Regarding purity parameters, A260/280 was significantly higher in samples with DNA yield above 100 ng. In addition, we performed amplicon-based targeted NGS on a subset of 20 samples. The most frequently mutated genes in the examined subset were KRAS, ERBB2, TP53, ATM and PALPB2. Our data are consistent with other similar data reported in the literature.

We performed a systematic review and meta-analysis to evaluate the prognostic role of cfDNA biomarkers in pancreatic adenocarcinoma. The results showed that cfDNA biomarkers indicate decreased overall survival and progression-free survival in PDAC, both in resectable and unresectable disease stages, and when detected during treatment.

Also, the result of the subgroup analysis dedicated to KRAS mutations detectable in cfDNA revealed a decrease in overall and progression-free survival in these patients when we included all stages of the disease in the analysis, but insignificant differences in survival between the two categories of patients in unresectable disease stages

On the other hand, the detection of circulating tumor DNA, through other methods than identification exclusive of KRAS mutations indicated decreased OS and PFS in unresectable PDAC cases.

The global risk of bias in the studies included in the systematic review was low for - reporting of statistical analysis, consideration of confounding factors, measurement of prognostic factors

and participation in the study, and moderate respectively for study dropout and outcome measurement.

We performed a second systematic review and meta-analysis evaluating the prognostic role of exosomal biomarkers in PDAC. Patients with positive exosomal biomarkers had decreased OS and PFS. Detection of exosomal biomarkers before surgery in resectable cases revealed an increased risk of progression. However, we did not detect a significant association between exosomal biomarkers and early mortality in unresectable disease stages. All investigated biomarkers are involved in tumor development and invasion processes. The overall risk of bias for both OS and PFS was low for - reporting of statistical analysis, measurement of confounding factors, assessment of study participation, measurement of outcomes and moderate respectively for study withdrawal. The risk of bias for the outcome measures was moderate for OS and low for PFS. The overall risk of bias was moderate-low for OS and for PFS it was low.

Conclusions

Molecular diagnosis in pancreatic adenocarcinoma, although currently applied in centers with high resources or predominantly for research purposes, will become indispensable for its correct management. When the disease is detected, in cases with an indication for treatment, obtaining a tumor sample will remain mandatory for establishing the diagnosis, but these samples will also be used to perform molecular tests. During the evolution of the disease, to evaluate the response to the treatment, and to monitor the changes in the tumor molecular profile, especially the mutational profile and therefore the opportunities to adapt the personalized therapy, it will be possible to perform liquid biopsy methods - the biological samples being obtained by minimal methods invasive, easily accepted by the patient and with affordable sampling costs. Performing high-throughput genetic testing such as next-generation sequencing has proved to be feasible in our study when using samples obtained by endoscopically guided fine needle aspiration puncture. In addition, through the performed meta-analyses, we highlighted a real potential to use liquid biopsy markers such as cfDNA and exosomal biomarkers for the assessment of prognosis in pancreatic adenocarcinoma and the follow-up of treatment response in specific clinical situations. Thus, the objectives of the research were achieved.

Regarding the technico-economic implications - the fact that FNA needles can also be used for acquiring samples dedicated to high-throughput genetic tests - is relevant. The current trend is for biopsy needles to replace cytology needles in ultrasound-guided sampling of solid pancreatic lesions. Biopsy needles, however, are still more expensive than cytology needles and are not widely available, especially in smaller centers. However, it will be essential to standardize the sample processing methods prior to the sequencing process to ensure comparable results of the genetic tests regardless of the method of sampling the material dedicated to this purpose.

Related to the use of liquid biopsy in PDAC to assess the prognosis of the disease - cfDNA biomarkers are predictors of survival in PDAC, and their positivity indicates the need for careful monitoring of patients. They indicate more aggressive tumor behavior and possible disease progression if detected during treatment. In resectable cases, they could help to make the decision regarding administration of neoadjuvant therapy. The detection of exosomal biomarkers in the blood of PDAC patients is associated with an increased risk of mortality, disease recurrence or resistance to chemotherapy. Although the vigilant monitoring of such cases seems justified, performing prospective studies in which therapeutic decisions also consider exosomal biomarkers are still necessary before developing clear recommendations regarding their use to guide the management of PDAC Lack of standard methods on the one hand of detecting this type of biomarkers and on the other hand of processing samples dedicated to this purpose and in addition, the inaccessibility of these methods (resulting from the high costs of access to high-performance equipment and dedicated personnel), currently prevent their routine use in the clinic Development of isolation kits – for both cfDNA and exosomes addresses both the need to reduce costs and to standardize the methods used.

The use of genetic testing to guide the management of PDAC in prospective randomized controlled trials including all disease stages is needed to strengthen current evidence and formulate international guideline recommendations. Especially for liquid biopsy biomarkers studies must include sufficiently long follow-up periods for a correct approximation of their impact on disease prognosis.

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