Plasma Circulating Tumor DNA in Pancreatic Cancer Patients Is a Prognostic Marker

Daniel Pietrasz1,2,3, Nicolas Pécuchet2, Fanny Garlan2, Audrey Didelot2, Olivier Dubreuil4, Solène Doat4, Françoise Imbert-Bismut5, Mehdi Karoui1,3, Jean-Christophe Vaillant1,3, Valérie Taïy2, Pierre Laurent-Puig2, and Jean-Baptiste Bachet2,3,4

Abstract

Purpose: Despite recent therapeutic advances, prognosis of patients with pancreatic adenocarcinoma remains poor. Analyses from tumor tissues present limitations; identification of informative marker from blood might be a promising alternative. The aim of this study was to assess the feasibility and the prognostic value of circulating tumor DNA (ctDNA) in pancreatic adenocarcinoma.

Experimental Design: From 2011 to 2015, blood samples were prospectively collected from all consecutive patients with pancreatic adenocarcinoma treated in our center. Identification of ctDNA was done with next-generation sequencing targeted on referenced mutations in pancreatic adenocarcinoma and with picoliter droplet digital PCR.

Results: A total of 135 patients with resectable (n = 31; 23%), locally advanced (n = 36; 27%), or metastatic (n = 68; 50%) pancreatic adenocarcinoma were included. In patients with advanced pancreatic adenocarcinoma (n = 104), 48% (n = 50) had ctDNA detectable with a median mutation allelic frequency (MAF) of 6.1%. The presence of ctDNA was strongly correlated with poor overall survival (OS; 6.5 vs. 19.0 months; P < 0.001) in univariate and multivariate analyses (HR = 1.96; P = 0.007). To evaluate the impact of ctDNA level, patients were grouped according to MAF tertiles: OS were 18.9, 7.8, and 4.9 months (P < 0.001). Among patients who had curative intent resection (n = 31), 6 had ctDNA detectable after surgery, with an MAF of 4.4%. The presence of ctDNA was associated with a shorter disease-free survival (4.6 vs. 17.6 months; P = 0.03) and shorter OS (19.3 vs. 32.2 months; P = 0.027).

Conclusions: ctDNA is an independent prognostic marker in advanced pancreatic adenocarcinoma. Furthermore, it arises as an indicator of shorter disease-free survival in resected patients when detected after surgery. Clin Cancer Res; 23(1); 116–23. ©2016 AACR.

Introduction

Pancreatic adenocarcinoma is a leading cause of cancer-related mortality in western countries and is predicted to become the second leading cause of cancer-related deaths in 2020 (1, 2). Surgery remains the cornerstone of treatment for patients with resectable pancreatic adenocarcinoma and the only curative treatment. The consequence of aggressive growth, early dissemination, and lack of early symptoms is that 80% of patients are diagnosed at late clinical stages (3). Despite recent improvements with new chemotherapy protocols, such as FOLFIRINOX or gemcitabine plus nab-paclitaxel (4, 5), patients’ prognosis remains very poor. Many serologic markers have been tested, like carbohydrate antigen 19-9 (CA 19-9), but none is highly prognostic in pancreatic adenocarcinoma (6). These tumors are characterized by tumor-specific genetic and epigenetic changes in DNA, including frequent mutations in CDKN2A, SMAD4, TP53, or KRAS genes (7–9), which can be used as potential markers. However, their widespread use is limited by the difficulty in obtaining tissues from patients using endoscopic techniques and because only 20% of patients present a resectable tumor. In this context, a prognostic noninvasive blood test for pancreatic adenocarcinoma would be very valuable.

Measuring tumor-specific alterations in blood nucleic acids offers an interesting approach. In this context, circulating tumor DNA (ctDNA) has produced interesting results for a wide range of cancers (10, 11). The fraction of patients with detectable plasmatic ctDNA as well as its concentration increased with tumor stage (12). Recent studies demonstrated the prognostic impact of ctDNA in metastatic colorectal cancer (13, 14). In fact, early changes in ctDNA during first-line chemotherapy have been shown to predict the later radiologic response (15).

Recent studies demonstrated a strong correlation between genomic alterations found in pancreatic tumors by sequencing and those found after characterization of DNA extracted from plasma of the same patients (16). ctDNA has also been evaluated...
Translational Relevance

The possibilities of translational research in pancreatic adenocarcinoma have been limited during recent years by the difficulty in obtaining sufficient tumor tissue, in quantity and quality. The identification of biological markers from blood could help to overcome this issue. Circulating tumor DNA (ctDNA) is one of the most promising blood biomarkers, providing information about molecular abnormalities and is associated with a prognostic and/or predictive value in others malignancies. Our study is the first to show that targeted NGS can be used for the detection of ctDNA in a routine procedure, across a large gene panel, and with concordant results obtained with picoliter droplet–based digital PCR. Our work highlights that the presence of ctDNA appears as an independent prognostic factor at all stages of pancreatic adenocarcinoma, in both advanced diseases and after curative intent resection. The described procedure offers great potential as a new, simple, and noninvasive strategy for patients’ care and follow-up.

Patients and Methods

Sample extraction and clinicopathologic data

From January 2011 to May 2015, plasmas of all consecutive patients with histologically proven pancreatic adenocarcinoma, receiving first-line chemotherapy protocol, were prospectively collected in the Pitié Salpêtrière Hospital (Paris, France), including resectable, locally advanced, and metastatic stages. Blood samples were collected just before (i) the first cycle of adjuvant treatment, after surgical resection in patients who had curative resection, or (ii) the first cycle of chemotherapy in patients with locally advanced or metastatic disease. All the patients signed an informed consent form, approved by the ethics committee (CPP Ile-de-France 2014/59NICB). The following data were collected in a prospective database: clinical and pathologic characteristics (gender, age, medical history, date of diagnosis, location of the primary tumor, primary tumor diameter, tumor differentiation grade, and stage of the disease), follow-up data (date of primary resection, date and type of relapse, date of diagnosis of metastatic disease, date and type of chemotherapy regimen, date and type of chemoradiotherapy, date of death, or last follow-up) and biological data (CEA, CA 19-9, albuminemia, and bilirubinemia).

DNA extraction from the plasma

Blood samples (9 mL) were withdrawn from a central catheter and placed in EDTA tubes. The collected samples were centrifuged at 3,500 rpm for 15 minutes at 4 °C within 3 hours of blood draw. Plasma was stored at −80 °C until further use. DNA was extracted from plasma with QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer’s instructions. Incubation with proteinase K was performed for 30 minutes at 68 °C. Extracted DNA from 2 mL of plasma was eluted with 50 μL buffer AVE and stored at −80 °C. DNA quantity was assessed using the Qubit dsDNA HS (high sensitivity) Assay Kit (Thermo Fisher).

Next-generation sequencing

Sequencing libraries were prepared from circulating-free DNA using Ion AmpliSeq Colon and Lung Cancer Research Panel v2 (Thermo Fisher; ref. 21). According to the manufacturer’s protocols, 10 ng of DNA for each sample was used as input for library preparation with the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher). The pooled barcoded libraries (maximum 96) were processed on Ion Chef System using the Ion PI Hi-Q Chef Kit (A27198) and sequenced on the Ion Proton System using an Ion PI Chip Kit v3 (A26771). The next-generation sequencing (NGS) analysis (see Supplementary Data File S1) has been specifically developed to detect low-allele frequency mutations, the sensitivity and specificity of which have been validated in positive and negative controls (22).

Droplet-based digital PCR

All plasma samples were screened for the three most frequent KRAS mutations in ctDNA with CA 19-9 levels, Dabritz and colleagues were able to diagnose pancreatic adenocarcinoma combining as a diagnostic marker to improve CA 19-9 value (17). By combining KRAS mutations in ctDNA with CA 19-9 levels, Dabritz and colleagues were able to diagnose pancreatic adenocarcinoma with a sensitivity of 91% (18). Despite these results, little information exists on the prognostic value of ctDNA (16, 19, 20).

The aim of the current study was to evaluate the feasibility of the detection of ctDNA and the prognostic value of ctDNA in patients with pancreatic adenocarcinoma.

cDNA Is a Prognostic Marker in Pancreatic Adenocarcinoma
any cause. Disease-free survival (DFS) was calculated from the date of surgery until first recurrence or death.

The Cox proportional hazards regression model was used to perform univariate and multivariate analyses with 95% confidence interval (CI). Multivariate analysis was performed with variables associated with the outcome in univariate analysis at a P value of < 0.1. All statistical analyses were performed using SPSS software version 21.0 (SPSS Inc.). A P value £ 0.05 was considered as significant.

Results
Study population
A total of 135 patients with resectable (n = 31; 23%), locally advanced (n = 36; 27%), or metastatic (n = 68; 50%) pancreatic adenocarcinoma were included in this prospective study (Table 1). Median age of patients was 65.6 years (range, 39.2–87.3). There was no difference between the three groups in demographic and biological characteristics except for baseline CA 19-9 (Table 1). Median age of patients was 65.6 years (range, 39.2–87.3). There was no difference between the three groups in demographic and biological characteristics except for baseline CA 19-9 (Table 1).

DNA sequencing
In patients with advanced pancreatic adenocarcinoma (n = 104), 50 (48%) of them had ctDNA detectable by at least one cancer-specific gene mutation with an MAF of 6.1% (range, 0.1–65.4). Of this group, 43 (41.3%) had a KRAS mutation (G12D n = 18; G12V n = 18; G12R n = 4; G12C n = 2; Q61H n = 5). TP53, SMAD4, NRAS, P13KCA, and STK11 gene mutations were detected in 23, 8, 2, 1, and 1 cases, respectively. In the group of patients with detectable ctDNA, 43 (86%) had a KRAS mutation, and 27 (54%) had at least two mutations detected (Fig. 1). ctDNA detection was strongly correlated to grade of tumor differentiation: 65% of patients in cases of undifferentiated tumors versus 58% in moderately differentiated versus only 30% in well-differentiated tumors (P = 0.036 and P = 0.042). Median CA19-9 was 380 IU/mL in patients with undetectable and 2,748 IU/mL in patients with detectable ctDNA (P = 0.015). Forty-four patients (64.7%) with metastatic disease had detectable ctDNA in comparison with only 6 patients (16.6%) with locally advanced pancreatic adenocarcinoma (P < 0.001). In the subgroup of metastatic patients, no significant correlation was found between the presence of ctDNA and the number of metastatic sites (P = 0.13).

In patients who had curative resection (n = 31), ctDNA was detected in 6 of them (19%), with a median MAF of 4.4% (range, 0.7–8.7). TP53 mutation was observed in 4 cases, KRAS and TP53 mutations in 1 case, and ERBB4 mutation in 1 case.

Correlation between NGS and dPCR
All KRAS mutations detected by NGS were confirmed by droplet-based dPCR in microfluidics. One patient, considered as negative after NGS, was detected positive with dPCR for the KRAS G12D mutation, with an MAF of 0.61%. Correlation between the two techniques revealed a high concordance with R² of 0.94 (Supplementary Data File S2).

Prognostic value of ctDNA in advanced pancreatic adenocarcinoma
After a median follow-up of 34.2 months, 76 patients died (73.1%). The presence of ctDNA was strongly correlated with
poor OS (6.5 vs. 19.0 months; log-rank $P < 0.001$) in patients with advanced pancreatic adenocarcinoma (Fig. 2A).

To evaluate the impact of ctDNA level, patients were grouped according to mutation allelic ratio tertiles. Patients with higher MAF had the worst OS (Fig. 2B). The OS decreased from 18.9, 7.8, and 4.9 months (log-rank $P < 0.001$) for the lowest, middle, and highest MAF tertiles, respectively. In multivariate analysis, including ctDNA, age, gender, and stage disease, ctDNA was independently associated with poor OS (HR = 1.96; 95% CI, 1.20–3.20; $P = 0.007$; Table 2).

Twenty-seven patients (54%) had at least two mutations detected. To evaluate the impact of presence of multiple mutations, patients were divided in two groups: "KRAS only" or "KRAS + other" when KRAS mutation was associated with at least another
mutation. There was a trend illustrative of patients with multiple mutations having a poorer prognosis, but the difference was not statistically significant (median OS 3.1 vs. 8.6 months; \( P = 0.128 \); see Supplementary Data File S3).

Similarly, in the subgroup of patients with a \( \text{KRAS} \) mutation, there was no significant difference in OS for particular individual \( \text{KRAS} \) mutations: \( \text{KRAS} \) G12V versus others: median OS 4.9 versus 9.0 months (\( P = 0.507 \)); \( \text{KRAS} \) G12D versus others: 4.9 versus 5.5 months (\( P = 0.594 \)).

Preliminary data from monitoring ctDNA in 8 patients suggest that the evolution of ctDNA levels was correlated with chemotherapy efficacy and objective radiological response (see Supplementary Data File S4).

Table 2. Prognostic factors for OS and DFS at univariate and multivariate analysis in advanced pancreatic adenocarcinoma

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>n = 104</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (male)</td>
<td>66</td>
<td>1.05 (0.99–10.4)</td>
<td>1.01 (0.97–10.4)</td>
</tr>
<tr>
<td>Metastatic disease</td>
<td>68</td>
<td>0.74 (0.45–1.19)</td>
<td>0.65 (0.38–1.36)</td>
</tr>
<tr>
<td>CA 19–9 &gt; 200 IU/mL</td>
<td>59</td>
<td>3.22 (1.90–5.45)</td>
<td>2.87 (1.52–5.42)</td>
</tr>
<tr>
<td>ctDNA detectable</td>
<td>50</td>
<td>1.33 (0.76–2.27)</td>
<td>1.16 (0.65–2.00)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.24 (1.41–3.54)</td>
<td>1.99 (1.13–3.50)</td>
</tr>
</tbody>
</table>

**NOTE:** Bold font indicates statistically significant \( P \) value.
*Continuous variable.

**Discussion**

In this series, we showed that ctDNA can improve the prognostic staging of metastatic and locally advanced pancreatic adenocarcinoma. Our work highlights that the detection and evaluation of the quantity of ctDNA appears suitable as an independent prognostic factor in stage III or IV pancreatic adenocarcinoma and a prognostic factor of recurrence in resected patients when detected after surgery.

Somatic mutations were analyzed in plasma DNA samples by NGS without any information on primary tumor mutational status. All \( \text{KRAS} \) mutations were also validated with picoliter droplet dPCR that provides both higher sensitivity and lower cost than NGS but is reduced in the number of mutations that can be interrogated. A strong correlation was observed between the AF measured by both approaches, suggesting that our NGS strategy led to qualitative results. In pancreatic cancer patients, the diagnostic benefit of optimized NGS compared with dPCR is approximately 15% of the cases (i.e., patients with a pancreatic cancer without \( \text{KRAS} \) mutations). The use of one technique or the other is likely to be linked to the workflow of the laboratory. Improvement of sensitivity is likely to be linked to testing of an increased quantity of plasma DNA. In dPCR, higher DNA input amounts could be used with an associated increase in sensitivity being achievable. We indeed previously described sensitivity of up to 0.0005%. However, only 6 ml of DNA was available for droplet-based dPCR analysis in this particular study. The use of a higher quantity of plasma DNA could have allowed the detection of a higher fraction of patients with \( \text{KRAS} \)-mutated ctDNA.

Bettegowda and colleagues reported that ctDNA somatic mutation can be detected in \( >50\% \) of patients with several cancers, including pancreatic adenocarcinoma, even in a localized stage (12). Moreover, Sausen and colleagues recently demonstrated that mutations detected in tumor specimens from 22 patients were detectable in the plasma at diagnosis with a specificity \( >99.9\% \), confirming that the mutated fraction of circulating DNA arises from tumor tissues (16). Although all our patients had histologically proven pancreatic adenocarcinoma, only 48% had detectable ctDNA in the advanced subgroup. Focusing on metastatic patients, this rate increased to 64.7%. These results are consistent with Kinugasa and colleagues’ cohort of 66 pancreatic adenocarcinoma patients screened for \( \text{KRAS} \) mutations, reporting 54.5% in single samples detectable for ctDNA in 6 tumor samples despite a significant number of metastatic cases in their cohort (15; ref. 19). However, Bettegowda and colleagues reported a higher ctDNA detection rate in metastatic PDAC with nearly \( 90\% \) of patients with detectable ctDNA. This discrepancy may be explained by methodologic differences and, more importantly, quantity of DNA sequenced in each assay in their study (19).

The development and growth of pancreatic adenocarcinoma involves oncogene activation or loss of tumor suppressor gene function (27, 28). \( \text{KRAS} \) is the most common of these genes and one of the drivers of mutations in pancreatic adenocarcinoma. Mutations in \( \text{KRAS} \) were the most frequently detected type in our cohort, in isolation or associated with other genes alterations, and present in 86% of patients with \( \text{ctDNA} \) detected. Our findings in blood are concordant with those previously published from pancreatic adenocarcinoma tissue, which report a rate of \( \text{KRAS} \) mutation in pancreatic adenocarcinoma of about 80% to 90% (16, 19). We retrospectively assessed the \( \text{KRAS} \) and TP53 tumor status in 20 tumor samples. We found an agreement between tumor tissue and plasma ctDNA in 19 of 20 couples tested (95%) both for \( \text{KRAS} \) and TP53. The 2 discordant cases (one for \( \text{KRAS} \) and one for TP53) were positive in plasma, negative in tumors, suggesting a potential sampling problem owing to biopsy from a unique metastatic site (data not shown).

Some authors demonstrated that gender, chronic inflammation, age, or tumor heterogeneity could influence the level of ctDNA (29). In this series, most patients with detectable ctDNA had moderate or undifferentiated tumors (\( P = 0.37 \)). Tumor differentiation thus seems to impact the ctDNA level in pancreatic adenocarcinoma. Although we did not observe a significant correlation between the number of metastatic sites and the presence of ctDNA, we cannot exclude a relationship between the tumor mass and the presence of ctDNA. The presence of a
pancreatic metastatic disease is not synonymous with presence of ctDNA, and seems to be correlated with differentiation grade and could more reflect tumor aggressiveness than tumor burden.

One of the important results in our study was the prognostic value of the presence and level of ctDNA. In the advanced subgroup, ctDNA was an independent prognostic biomarker of OS (HR = 1.94; \( P = 0.007 \)). The presence of ctDNA after surgery appeared to be a prognostic factor of poor DFS and OS. Chen and colleagues have previously described the prognostic value of this biomarker in pancreatic adenocarcinoma (20). In their series, the presence of \( \text{KRAS} \) mutation in plasma was correlated with poor OS (3.9 vs. 10.2 months; \( P < 0.001 \)) in nonresectable patients. More recently, Sausen and colleagues reported that, in resectable patients, ctDNA was a prognostic factor of early tumor relapse if detected before surgery (log-rank \( P = 0.015 \)). In this study, in a subgroup of 20 patients collected after surgical resection, detectable ctDNA was also a prognostic biomarker of DFS (9.9 months vs. median not reached; log-rank \( P = 0.02 \); ref. 16).

Although, in resected patients, the presence of ctDNA after resection could suggest the existence of a micrometastasis disease, studies are needed to determine whether the presence of ctDNA may be used for early detection of pancreatic adenocarcinoma. On the basis of our results, we make an educated guess that the detection of small pancreatic tumors will be difficult without improving the quantity of input DNA and consequently the volume of plasma used for this purpose.

Figure 3.
Survival curves according to detectable ctDNA in resected patients. A, Disease-free survival. B, Overall survival.
Although the blood samples of our patients are prospectively and consecutively collected, the study presents heterogeneity in treatments received owing to difference in age, treatment tolerance, or performance status of patients. This heterogeneity could induce a bias in the results even if multivariate analysis confirmed the prognostic value of ctDNA. Second, our monitoring results are preliminary without perfect concordance between sample collection and radiological evaluation of patients for example. In the resectable subgroup, there is a lack of preoperative ctDNA data. Indeed, patients were collected at the first cycle of adjuvant treatment, and our protocol did not include samples before surgery. Finally, despite careful collection and storage of samples, and the use of highly sensitive methods to detect genetic changes, some alterations may not have been detected due to limited plasmatic DNA amounts or very low MAF.

Conclusions
This study demonstrates that ctDNA can be detected in peripheral blood in pancreatic adenocarcinoma and that it appears as an independent prognostic marker of OS in locally advanced or metastatic diseases. Furthermore, it arises as an indicator of shorter DFS and OS in resected patients when detected after surgery. The described procedure may have great potential as a new simple and noninvasive strategy for patients’ care and follow-up. Further investigations are needed to confirm these results and their usefulness in the prognosis and in-risk group screening of patients.

Moreover, ctDNA would be of great interest (i) to allow a rapid molecular analysis for inclusion in "molecular" trials with targeted therapies; and (ii) for initial diagnosis in the case of difficulty in obtaining histologic proof.

Disclosure of Potential Conflicts of Interest
V. Taly is a consultant/advisory board member for Boehringer Ingelheim and Raindance Technologies. P. Laurent-Puig is a consultant/advisory board member for Raindance Technologies. J.-B. Bachet is a consultant/advisory board member for Amgen, Celgene, and Merck Serono. No potential conflicts of interest were disclosed by the other authors.

References


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