Research Article

Early Relapse Detection and Monitoring Disease Status in Patients with Early-stage Pancreatic Adenocarcinoma using Circulating Tumor DNA

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Simple Summary

This study aimed to evaluate and validate the relationship between circulating tumor DNA (ctDNA) and recurrence in pancreatic adenocarcinoma while evaluating the relationship between tumor markers (CEA and CA19-9) and ctDNA. Furthermore, the study evaluated molecular residual disease clearance as

assessed by ctDNA in the neoadjuvant and adjuvant therapy settings for pancreatic adenocarcinoma patients.

Nine patients with pancreatic cancer were enrolled (8 patients with pancreatic cancer and 1 patient with ampullary adenocarcinoma). The ctDNA samples were collected during the patients' follow-up visits, with a median of 357 days (interquartile range: 248-542 days). Additional monitoring included carcinoembryonic antigen (CEA), cancer antigen 19-9 (CA 19-9), and radiological imaging. Personalized mutational profiles derived from tumor tissue via whole-exome sequencing were used to design patient-specific ctDNA assays for variant detection in plasma samples (Signatera test, Natera). Our findings suggest that the presence of ctDNA after surgery in early-stage PDAC is associated with reduced recurrence-free survival. During monitoring, ctDNA was found to be a better prognostic marker compared to CA-19 9 and CEA and it can be used to inform on disease status prior to imaging.

Abstract

Pancreatic ductal adenocarcinoma (PDAC) has one of the most aggressive cancer histologies, with high recurrence (85%) and a 5-year survival rate of 9%. The circulating tumor DNA (ctDNA) is evolving field in setting of management for patients with advanced solid tumors. In this study, our aim was to investigate the potential contributions of ctDNA in early-stage pancreatic adenocarcinoma prognostication as early relapse detection and disease status monitoring and to evaluate the relationship with tumor markers carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA 19-9).

Methods: We enrolled 9 patients with pancreatic cancer from whom we collected paired tissue and plasma/serum samples. We analyzed the ctDNA of patients, with a median follow-up of 357 days, and monitored CEA and CA19-9, and radiological imaging. Personalized mutational profiles derived from tumor tissue via whole-exome sequencing were used to design patient-specific ctDNA assays for variant detection in plasma samples (Signatera test, Natera). Results: We found that during the course of the follow-up, 44.4% (4/9) of patients relapsed. The presence of ctDNA was associated with reduced recurrence-free survival (p = 0.011). Importantly, ctDNA results were found to correlate and precede imaging results. In contrast, CA 19-9 and CEA, in certain cases showed discordance with imaging and were found to be elevated due to other benign conditions, such as gastritis.

Conclusion: This study demonstrates that presence of ctDNA after surgery in early-stage PDAC is associated with reduced recurrence-free survival. During monitoring, ctDNA was found to be a better prognostic marker compared to CA-19-9 and CEA and can be used to inform on disease status prior to imaging.

Introduction

Pancreatic adenocarcinoma (PDAC) has one of the most aggressive cancer histologies, with high recurrence (85%) and a 5-year survival rate of 9% [1,2]. Frequently, at the time of diagnosis curative surgery is not a treatment option. For these cases of locally advanced or metastatic pancreatic cancer, Fluorouracil-based and gemcitabine-based

chemotherapy is the usual treatment and despite significant improvements in combination treatment for pancreatic cancer, the 5-year survival rate for these patients is only 5% [2,3]. CA19-9 levels are commonly utilized in clinical settings to predict prognosis and to evaluate tumor response to pancreatic cancer treatments, but CA19-9 levels may be abnormally high in patients with benign non-malignant conditions like biliary obstruction or infection and may be undetectable in Lewis antigen-negative individuals.

Circulating tumor cells (CTCs) have recently emerged as a possible biomarker. CTCs are tumor cells shed from primary or metastatic sites that reach the peripheral circulation. Mandel and Metais were identified cell-free DNA in blood plasma for the first time in 1948 [4], and it was also determined in 1965 and 1966 that cell-free DNA might be relevant in metastasis and illness. In addition, individuals with systemic lupus erythematosus had higher quantities of cell-free DNA [5,6]. Leon and colleagues performed radioimmunochemistry to show that there was an increase in cell-free DNA in cancer patients compared to normal control participants about three decades after it was initially found [7]. Due to technical difficulties, cell-free DNA was not revealed to have directly generated from tumor cells until the mid of 90s [8,9]. Two experiments published in 1994 discovered tumor-specific mutations in plasma samples from patients with pancreatic adenocarcinoma and acute myelogenous leukemia. In these experiments, mutation-specific primers were utilized to amplify N-RAS mutations using polymerase chain reaction (PCR). Normal DNA in body fluids dilutes CtDNA, which is commonly produced from CTCs and/or original tumor cells. As a consequence, conventional sequencing methods in the 1990s, such as Sanger sequencing, weren't sensitive enough to discover mutant ctDNA molecules. The only method that allowed for greater identification of the weak tumor-specific cell-free DNA signal was mutation-specific PCR. Until the development of large parallel sequencing/next generation sequencing technologies, this technique was the favored way for assessing mutation status in cell-free DNA [10]. By assessing fetal DNA in maternal plasma, cell-free DNA began to be clinically utilized in prenatal diagnosis of sex determination and pregnancy-related diseases from 1997 to 2000 [11-13]. Beads, emulsion, amplification, magnetic was a digital PCR method used to identify and quantify mutations in the plasma of patients with colorectal cancers in 2005 [14]. According to this study, patients with advanced colorectal tumors exhibited mutant adenomatous polyposis coli (APC) DNA molecules in their plasma, Mutant APC DNA molecules were found at quantities ranging from 0.01 to 1.7 percent of total APC molecules in >60 percent of patients with early and potentially curable colorectal cancer, indicating that tumor cell DNA released in the blood can be used for diagnostic tool. Since the development of digital PCR and NGS-based technologies to detect ctDNA, ctDNA has become a biomarker in a variety of cancers, including lymphoma, thyroid cancer, breast cancer, gastrointestinal stromal tumors, colorectal cancer, and lung cancer (but not pancreatic cancer yet), and it's especially useful for detecting common mutations in genes for example BRAF, EGFR, PIK3CA, KRAS, P53 etc [15-24]. Due to these promising earlier studies, ctDNA has now been utilized in clinical trials, particularly because ctDNA analysis will allow clinical investigators to monitor tumors in response to targeted therapy, monitor development of resistance, and detect minimal residual disease [25-27]. Recently, numerous circulating biomarkers have been evaluated for the

detection of molecular residual disease (MRD) and molecular monitoring in PDAC. More recent studies have demonstrated the usefulness of ctDNA in minimal residual disease surveillance in HCC [28-30]. For example, Kasi et al, analyzed 200 plasma samples from a total of 90 hepatobiliary patients, with 27 of these patients having HCC [31]. It was reported that ctDNA detection was significantly associated with the stage of disease. In addition, serial time point analysis was performed on a subset of patients (n=56) that had 2-7 time points available and correlations between ctDNA levels and clinical response were noted and presented [29,31,32]. However, the application of these techniques has been limited in early-stage PDAC due to poor sensitivity and specificity [2]. More recently, an ultrasensitive, personalized, and tumor-informed ctDNA assay has shown to overcome many of the challenges that have plagued the aforementioned biomarkers, thus allowing for reliable detection of MRD across different tumors.

Materials and Methods

This single center study retrospectively reviewed patients with pancreatic cancer at the Houston Methodist Cancer Center who have data available for ctDNA testing. A total of nine metastatic pancreatic cancer patients were retrospectively reviewed for ctDNA analysis between May 2019 and March 2021. Reviewing patients charts, all plasma samples were collected from 8 patients with pancreatic cancer and 1 patient with ampullary adenocarcinoma postoperatively prior to and after adjuvant therapy per patients follow up office visits and surveillance scans.

For all patients, data on post-surgery clinical intervention and other clinicopathologic information were obtained and followed up for a median of 357 days (interquartile range: 248-542 days). In order to

confirm the diagnosis, the pathology of these patients was determined using biopsy samples obtained by the oncological surgeons of Houston Methodist Cancer Center. All patients were treated and followed up in accordance with The National Comprehensive Cancer Network (NCCN) recommendations.

Personalized mutational profiles derived from tumor tissue via whole-exome sequencing were used to design patient-specific ctDNA assays for variant detection in plasma samples. Multiplex PCR primer pairs for the selected set of variations were created. Cell-free DNA was isolated from a median of 8.5 mL of plasma (interquartile range, 7.5-9-5 mL). As previously reported, A-tailing, and ligation using an adapter were used to generate universal libraries, and following that, libraries were amplified using multiplex PCR, barcoded, pooled, and sequenced on an NGS sequencing platform (HiSeq2500 system, Illumina Inc). ctDNA plasma samples were classified as positive if they had at least two variants identified per provider (Signatera test, Natera).

Apart from ctDNA analysis, patients were also monitored using carcinoembryonic antigen (CEA), cancer antigen 19-9 (CA 19-9), and radiological imaging. In addition, computed tomography (CT) or Magnetic resonance imaging (MRI) was used to evaluate the treatment response every 8-12 weeks according to the guidelines of Response Evaluation Criteria in Solid Tumors (RECIST) 1.0.

Recurrence-free survival (RFS) was the major outcome measure, as determined by conventional radiologic criteria. Recurrence-free survival was calculated from the date of surgery to the first confirmed radiologic recurrence (local or distant) or death due to pancreatic cancer and was censored at the final follow-up or non-pancreatic cancer- related death. The Kaplan-Meier analysis was performed between ctDNA positive and

negative groups, and between CA19-9 elevated and normal groups. Log-rank test was used to test the overall survival between the two groups. Statistical significance was defined as two-tailed p<0.05 for all tests. Stata IC/12.1 (StataCorp) and R statistical software, version 2.4 for Windows, were used to conduct the statistical analysis (R Foundation for Statistical Computing). Natera Inc performed the ctDNA analysis retrospectively, with scientists blinded to patient outcome and sample order.

Patient ID	ctDNA Status	Sex	Age	CA 19-9 Status	Surgery Date	Relapse	Date of Recurrence or Last follow up visit
1	Negative	F	63	Elevated	4/17/2019	No	7/15/2021 Last follow up
2	Positive	F	77	Elevated	02-12-2020	Yes	11/17/20 Recurrence
3	Positive	M	42	Elevated	06-06-2019	Yes	11/29/20 Recurrence
4	positive	F	49	Elevated	09-02-2020	Yes	2/9/21 Recurrence
5	Negative	F	70	Normal	03-11-2020	No	7/15/2021 Last follow up
6	Negative	M	63	Normal	09-10-2019	No	7/22/2021 Last follow up
7	Positive	F	59	Elevated	05-05-2020	Yes	1/8/21 Recurrence
8	Negative	M	71	Normal	7/14/2020	No	7/6/2021 Last follow up
9	Negative	M	83	Normal	01-05-2021	No	7/1/2021 Last follow up

Table 1: ctDNA: Circulating tumor DNA; CA19-9: Cancer antigen 19-9. Patinates highlighted with in green had negative ctDNA that is associated with no recurrences of cancer, while all the patinates highlighted in red had positive ctDNA test that is associated with disease relapse.CA 19-9 was elevated in patient#1 however, no recurrence was reported.
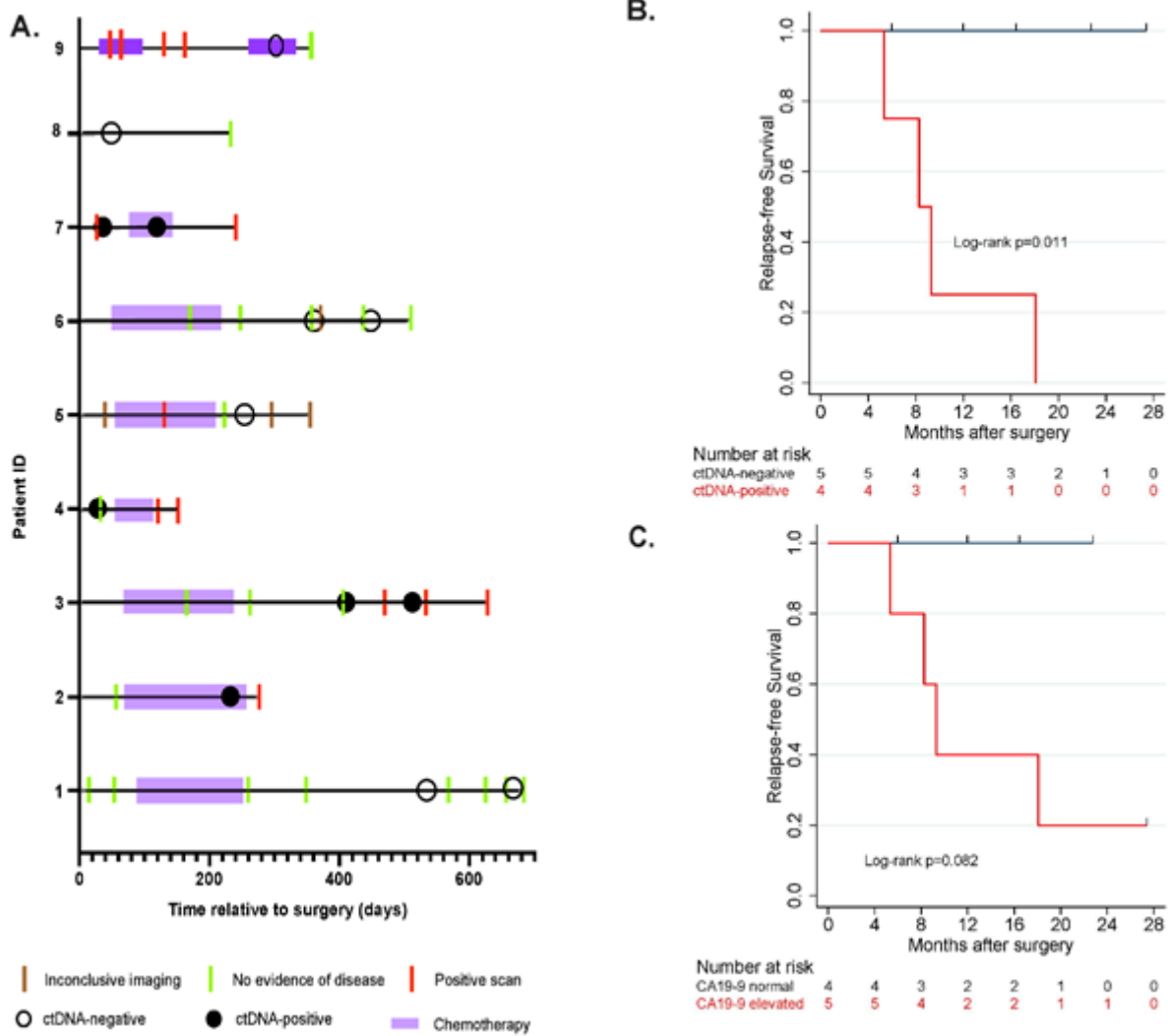
Figure 1: A. Patient over view plot, B,C. association of relapse- free survival with ctDNA and CA-19-9 status in longitudinal setting

1A: In this cohort, post- surgical ctDNA analysis detected relapse in 4 patients with a median follow up of 357 days (interquartile range: 248-542 days). B,C: Present the estimated relapse- free survival distributions by ctDNA and CA 19-9. The result of log rank test indicates significant differences between the survival curves between patints with ctDNA positive with ctDNA negative (p=0.011). the survival curves between patients with CA 19-9 elevated and CA 19-9 normal were not significant different (p=0.082). Moreover, CA 19-9 and CEA showed discordance with imaging and were elevated due to other conditions, such as gastritis.
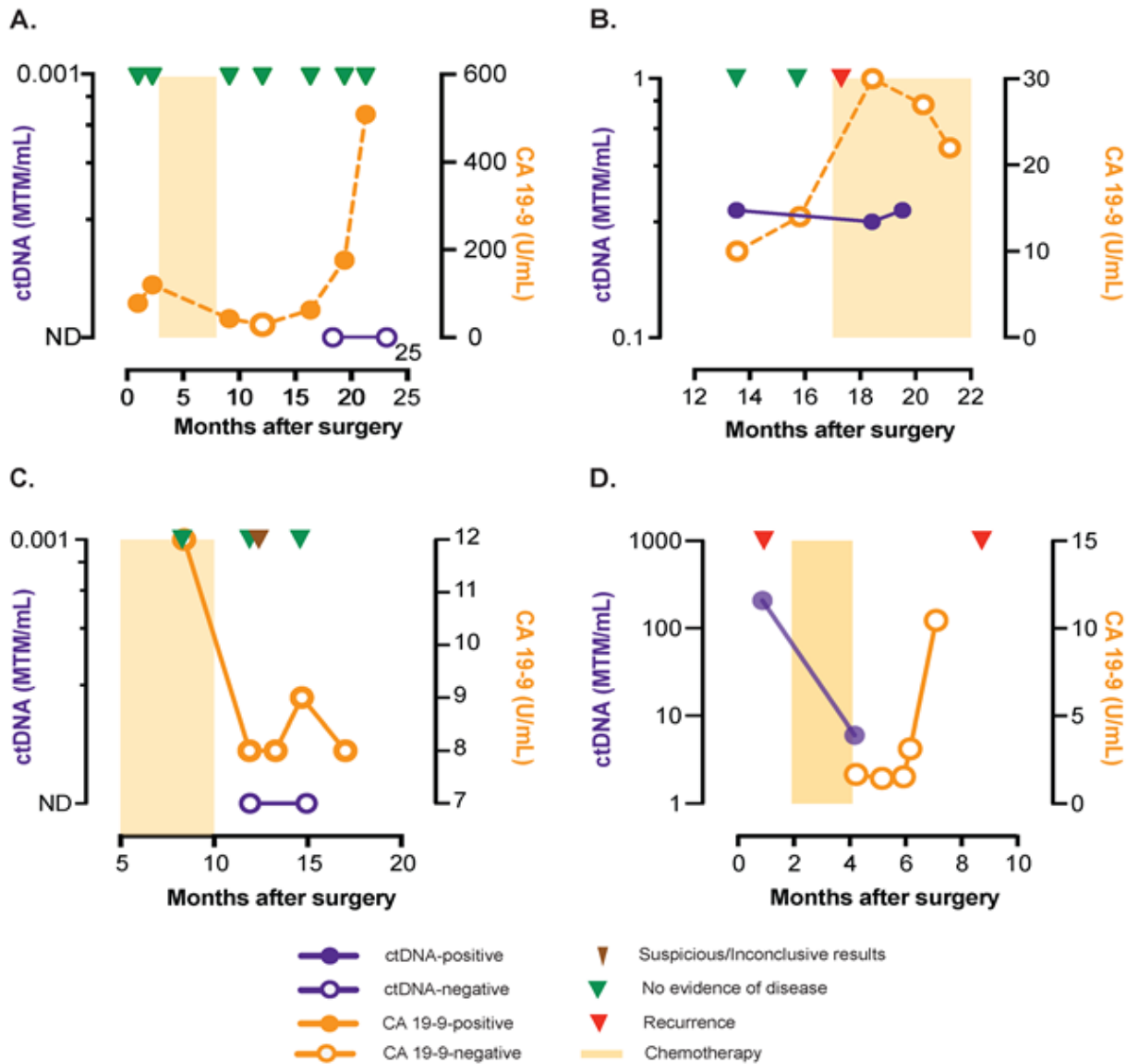
Figure 2: ctDNA for early relapse detection and disease status monitoring

Figure 2A-D: During monitoring, ctDNA is a better prognostic marker compared to CA-19-9.

B,D: Three patients had collected plasma after surgery, of which 2 patients (MRD rate 66%) were found to be ctDNA-positive with both experiencing relapse. MTM: Mean tumor molecules.

Results

Circulating tumor DNA associated with minimal residual disease and relapse

In this retrospective study, post-surgical ctDNA analysis detected relapse in 4 patients with a median follow-up of 357 days (interquartile range: 248-542 days; Figure 1A). During follow-up, 44.4% (4/9) of patients relapsed and of these patients, 100% had ctDNA detected prior to or at the time of recurrence (100% sensitivity and specificity. Presence of ctDNA (Figure 1B) was significantly associated with reduced relapse-free survival (log-rank p=0.011). In comparison, CA 19-9 (Figure 1C) showed inferior performance in predicting relapse. The survival curves

between patients with CA19-9 elevated and CA19-9 normal were not significant different (p=0.082). Moreover, CA-19-9 and CEA showed discordance with imaging and were elevated due to other conditions, such as gastritis. During monitoring, ctDNA was a better prognostic marker compared to CA-19-9 (Figure 2A - D). Three patients had plasma collected after surgery and 2 of these patients (Minimal residual disease MRD rate of 66%) were found to be ctDNA-positive with both patients experiencing relapse (Figure 2B, D).

Table 1 showed that all patinates with negative ctDNA results (highlighted in green) showed no recurrences even with falsely elevated Ca19-9 (patient #1), while patinates with positive ctDNA values (highlighted in red) showed disease recurrence.

Circulating tumor DNA associated with disease status: Patients' presentations

Figure 3 highlights patients' presentations and demonstrates that ctDNA test result can be associated with disease status more accurately that representative tumor markers.

In patient number 1, who was diagnosed with pancreatic cancer in April 2019 and then had surgery in the same month, surveillance post operation CT scans revealed no evidence of disease (NED). Adjuvant chemotherapy (ACT) was started in July 2019 and continued until December 2019. CT was performed several times during 2020 and showed NED. Similarly, ctDNA analysis done in October 2020 showed a negative value. During the same time period, in October 2020, CA19-9 was detected at a high level but following EDG the CA19-9 elevation was linked to gastritis. These results suggest that ctDNA may be a better prognostic marker than CA-19-9 for pancreatic cancer. In patient 5, ctDNA was negative in November

2020 and the subsequent CT scans showed NED around the same time. Similarly, in patient 6, the value of ctDNA was connected to the disease course where ctDNA revealed a negative value in September and December 2020 and CT scans indicated NED during the same time period. For Patient 7, who had Whipple surgery in May 2020, CT scans performed in June 2020 showed the presence of residual disease. At the same time, ctDNA analysis matched the diagnosis. Patient 9 was diagnosed with pancreatic cancer in June 2020, received FOLFIRINOX from June 2020 to September 2020. The imaging was repeated in October 2020, confirming persistence of disease ctDNA was negative in April 2021 and subsequent CT scan performed in May 2021 was consistent with this result (Figure 3).

Circulating tumor DNA informs prior to surveillance imaging:

Regarding patient 2, who diagnosed in January 2020 and had Whipple surgery in February 2020 the CT revealed NED on April 2020. The patient started to receive mFOLFIRINOX and was then switched to Gemcitabine/ oxaliplatin in July 2020.The ctDNA showed positive results in October 2020 prior to the CT done in November 2020 that informed of recurrence (Figure 4).

Interestingly, in-patient number 3, who had Whipple surgery in June 2019, imaging showed NED until July 2020, whereas ctDNA analysis performed at the same time, revealed a positive value indicative of recurrence. At a later time, in November 2020, CT confirmed the recurrence demonstrating that ctDNA was informing us of recurrence in advance to imaging., Since pancreatic cancer is an extremely aggressive disease, early markers of disease progression are of great value for appropriate treatment decisions (Figure 4). The

ctDNA of Patient 4 detected the recurrence in September 2020 while the CT scan showed NED until January 2021. After that date, the CT confirmed the recurrence. These findings suggest that ctDNA can detect earlier signs of illness that could aid in critical treatment decisions Patient 8 had a pancreatic mass diagnosed in June 2020, and then Whipple surgery in July 2020. In September 2020, ctDNA revealed negative results prior to the CT scan performed in March 2021 that confirmed NED (Figure 4).
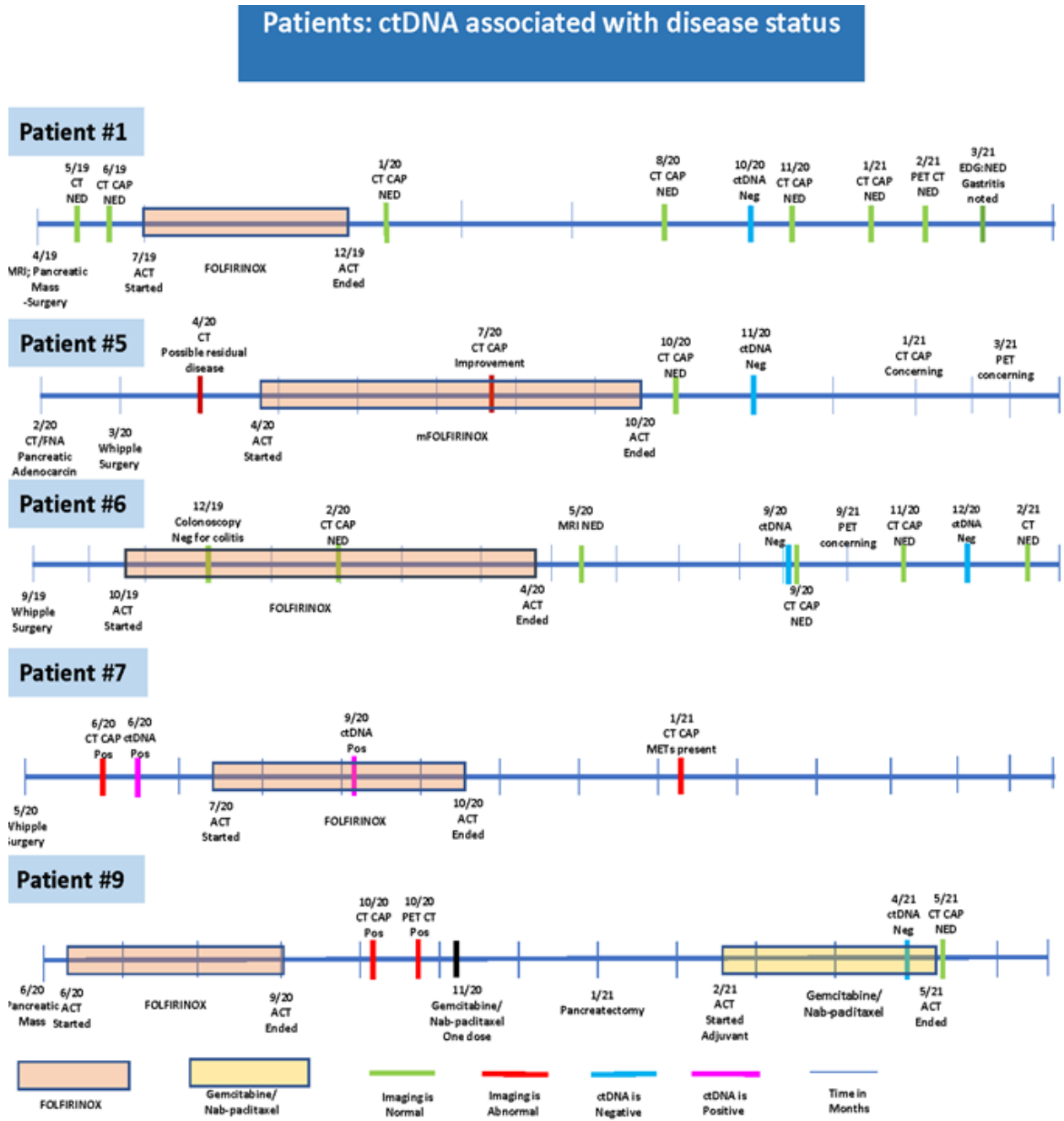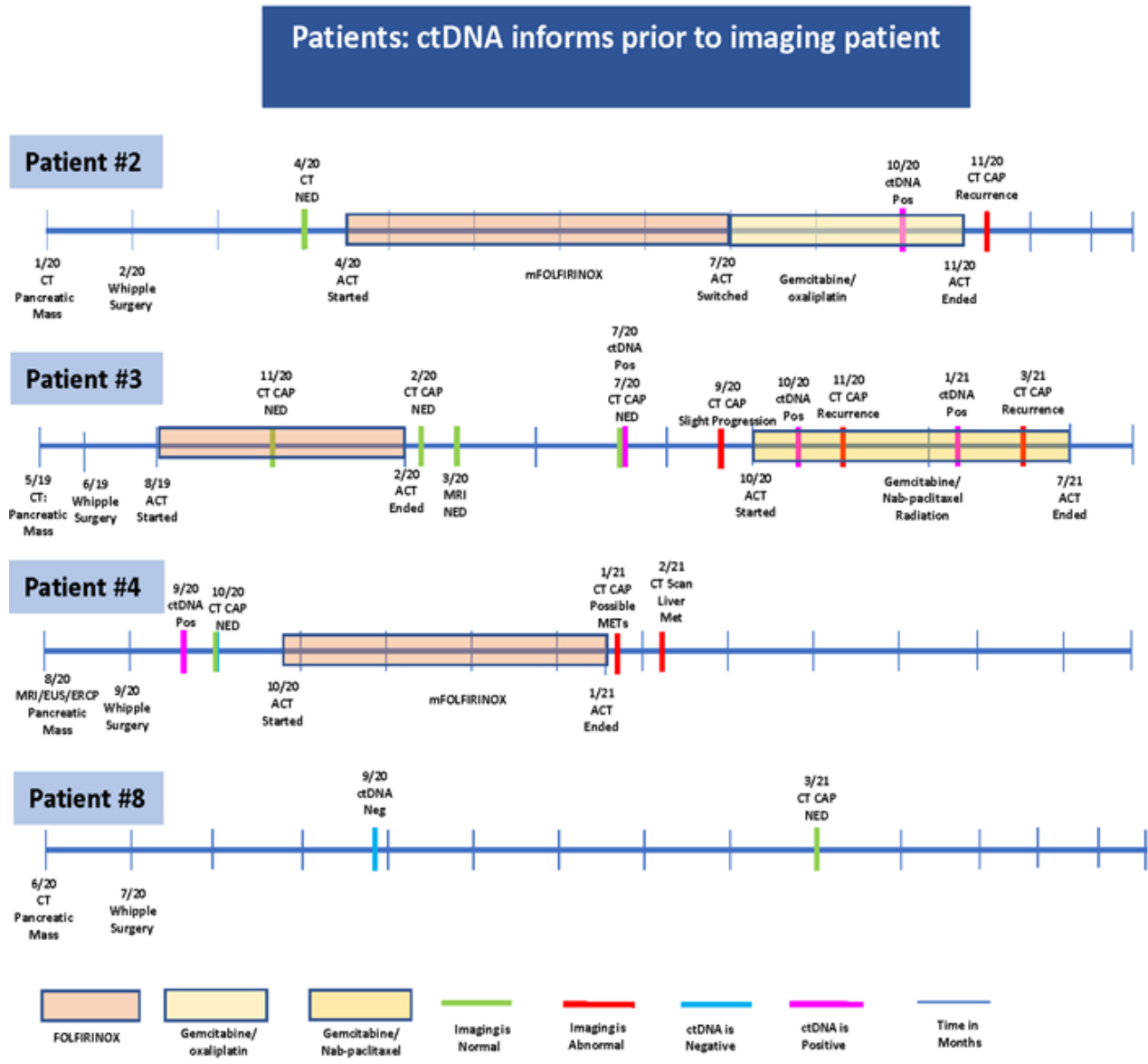

Figure 3: **CT:** computerized tomography; **CAP:** chest, abdomen and pelvis; **MRI:** magnetic resonance imaging; **PET:**

positron emission tomograohy; **EUS:** endoscopic ultra sound; **ERCP:** endoscopic retrograde cholangiopancreatography; **NED:** no evidence of disease; **act:** a chemotherapy; **ctDNA:** circulating tumor DNA; **EDG:** esophagogastroduodenoscopy; **Neg:** negative; **Pos:** positive; **METs:** metastasis; **FNA:** fine needle aspiration

Figure 4: CT: computerized tomography; **CAP:** chest, abdomen and pelvis; **MRI:** magnetic resonance imaging; **PET:** positron emission tomograohy; **EUS:** endoscopic ultra sound; **ERCP:** endoscopic retrograde cholangiopancreatography; **NED:** no evidence of disease; **act:** a chemotherapy; **ctDNA:** circulating tumor DNA; **EDG:** esophagogastroduodenoscopy; **Neg:** negative; **Pos:** positive; **METs:** metastasis; **FNA:** fine needle aspiration

Discussion

The analysis of ctDNA represents a unique method to explore the DNA in the bloodstream that comes from cancerous cells and tumors the clinical arena. To our knowledge, this is one of the first retrospective study to investigate the validity of using ctDNA test in pancreatic cancer patients in correlation with tumor markers such as CA19-9 and imaging. One of the first stages in establishing a cancer diagnosis is to do a complete molecular examination of tissue biopsy samples. However, due to the difficulties in collecting sufficient tissue samples in patients with pancreatic cancer, the clinical value of such a study is typically decreased. As a result, there has been a lot of attention on developing and implementing highly accurate and less invasive cancer monitoring blood tests. Although various serological biomarkers are utilized in the clinic for pancreatic patients, such as CEA and CA 19–9, they are neither sensitive nor specific enough for prognostication. It has shown that ctDNA is produced from the original tumor and/or its metastases, and that it can be detected using next-generation sequencing or ddPCR with great sensitivity(33, 34). As a result, it might be utilized for cancer surveillance and minimal residual disease evaluation.

The ctDNA biopsy offers a noninvasive approach that potentially resolves the limited access to pancreatic cancer tissue samples by tissue biopsy. In addition, ctDNA biopsy reveals a dynamic picture of pancreatic cancer disease status, easily repeatable when needed, and provides real-time surveillance for minimal residual disease and long-term monitoring in pancreatic cancer patients that is more informative than the current tumor markers.

In our study, a total of nine patients with resectable pancreatic or ampullary adenocarcinoma who had ctDNA data available were retrospectively analyzed.

The mean age was 64.1 ± 13.0 years and the male population represented 44.4 % (n=4). All plasma samples from 9 patients with a median follow-up of 357 days (interquartile range: 248-542 days) were analyzed and quantified using ultradeep multiplex PCR–based NGS per provider (Signatera test, Natera). Eight patients received mFOLFIRINOX adjuvant chemotherapy, two of them received additional rounds of gemcitabine with Nab-paclitaxel and one received gemcitabine with oxaliplatin. Three patients had plasma collected after surgery and 2 of these patients were found to be ctDNA-positive and eventually relapsed. During the course of the follow-up, 44.4% (4/9) of patients relapsed (Table 1). The presence of ctDNA was associated with reduced recurrence-free survival ($p = 0.011$). Circulating tumor DNA findings were found to correlate and precede imaging results (Figure 4). In contrast, CA 19-9 and CEA levels, in certain cases, showed discordance with imaging results and were found to be elevated due to other benign conditions, such as gastritis.

We found during monitoring, ctDNA is a better prognostic marker compared to CA-19-9 (Figure 2A-D). Three patients had plasma collected after surgery, of which 2 patients (MRD rate of 66%) were found to be ctDNA-positive with both experiencing relapse. (Figure 2B, D)

While this stay is a retrospective study on small number of patients, it suggests and highlights the clinical utility of ctDNA in an aggressive GI cancer like pancreatic adenocarcinoma that is known for short recurrence. A cohort study in manner of prospective follow-up and successive blood collection may be necessary in the future to demonstrate the efficacy of using ctDNA as biomarker for pancreatic cancer MRD and surveillance which is our next planned prospective study.

Conclusions

Our findings suggest, presence of ctDNA after surgery in early-stage PDAC is associated with reduced recurrence-free survival. During monitoring, ctDNA was found to be a better prognostic marker compared to CA-19 9 and CEA and can be used to inform on disease status prior to imaging. More studies are needed to evaluate using ctDNA as biomarker for pancreatic cancer MRD and surveillance which is our next planned prospective study.

Author Contributions

Abdelrahim M, Esmail A, contributed to the conception; Abdelrahim M , Esmail A and Malhotra M contributed to abstract writing; Abdelrahim M, Esmail A, contributed to literature search and acquisition; Abdelrahim M, Esmail A are responsible for drafting and revising the manuscript; Abdelrahim M, Esmail A, Xu J, Tiffany A. Katz T, Sharma S, Kalashnikova E, Malhotra M, Olshan P, Billings P and Aleshin A contributed to analysis and interpretation; Abdelrahim M was the patients oncologist and was responsible for the critical revision of the manuscript for intellectual content; and all authors issued final approval for the version to be submitted. All authors affirm final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Acknowledgments and Disclosures

Conflicts of interest/Financial disclosures

None reported. All authors have declared there are no financial conflicts of interest with regard to this work.

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