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Mutant *KRAS* Circulating Tumor DNA Is an Accurate Tool for Pancreatic Cancer Monitoring

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Pancreatic cancer • Adenocarcinoma • *KRAS* • Circulating tumor DNA • Liquid biopsy • Cancer monitoring

Abstract

Background. Many new pancreatic cancer treatment combinations have been discovered in recent years, yet the prognosis of pancreatic ductal adenocarcinoma (PDAC) remains grim. The advent of new treatments highlights the need for better monitoring tools for treatment response, to allow a timely switch between different therapeutic regimens. Circulating tumor DNA (ctDNA) is a tool for cancer detection and characterization with growing clinical use. However, currently, ctDNA is not used for monitoring treatment response. The high prevalence of *KRAS* hotspot mutations in PDAC suggests that mutant *KRAS* can be an efficient ctDNA marker for PDAC monitoring.

Subjects, Materials, and Methods. Seventeen metastatic PDAC patients were recruited and serial plasma samples were collected. CtDNA was extracted from the plasma, and *KRAS* mutation analysis was performed using next-generation sequencing and correlated with serum CA19-9 levels, imaging, and survival.

Results. Plasma *KRAS* mutations were detected in 5/17 (29.4%) patients. *KRAS* ctDNA detection was associated with shorter survival (8 vs. 37.5 months). Our results show that, in ctDNA positive patients, ctDNA is at least comparable to CA19-9 as a marker for monitoring treatment response. Furthermore, the rate of ctDNA change was inversely correlated with survival.

Conclusion. Our results confirm that mutant *KRAS* ctDNA detection in metastatic PDAC patients is a poor prognostic marker. Additionally, we were able to show that mutant *KRAS* ctDNA analysis can be used to monitor treatment response in PDAC patients and that ctDNA dynamics is associated with survival. We suggest that ctDNA analysis in metastatic PDAC patients is a readily available tool for disease monitoring. ***The Oncologist*** 2018;23:566–572

Implications for Practice: Avoiding futile chemotherapy in metastatic pancreatic ductal adenocarcinoma (PDAC) patients by monitoring response to treatment is of utmost importance. A novel biomarker for monitoring treatment response in PDAC, using mutant *KRAS* circulating tumor DNA (ctDNA), is proposed. Results, although limited by small sample numbers, suggest that ctDNA can be an effective marker for disease monitoring and that ctDNA level over time is a better predictor of survival than the dynamics of the commonly used biomarker CA19-9. Therefore, ctDNA analysis can be a useful tool for monitoring PDAC treatment response. These results should be further validated in larger sample numbers.

Introduction

Pancreatic cancer is the fourth most common cause of cancer death among men and women in the U.S. [1]. Although it is not a common disease, ranked 11th in incidence in men and 9th in women [1], the high mortality rate of this disease leads pancreatic cancer-associated death to be a significant burden on society. The burden of this disease is constantly growing, mostly due to the growing obesity epidemic [2]. The high mortality rate of pancreatic cancer stems from late diagnosis—53% of patients are diagnosed with a metastatic disease. Patients who are diagnosed with metastatic disease have a 5-year overall survival rate of 2% [3]. Therefore, methods for early detection and novel treatment modalities are a significant unmet need.

Although the prognosis of this disease is still grim, several new pancreatic cancer treatments have been approved in this decade. Until recently, gemcitabine was the mainstay of treatment for metastatic disease, with marginal additional benefit from gemcitabine combinations [4, 5]. In 2011, a combination of three chemotherapeutic agents—fluorouracil, oxaliplatin, and irinotecan (FOLFIRINOX)—nearly doubled metastatic pancreatic cancer survival [6]. Shortly thereafter, the addition of

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nab-paclitaxel to gemcitabine showed significant improvement in overall survival, with a much better side-effect profile compared with FOLFIRINOX [7]. Therefore, different treatment combinations are currently available to metastatic pancreatic cancer patients, and it is important to switch between them wisely. The growing number of treatment options for pancreatic cancer patients, combined with the significant toxicities of current treatments, raises the need for better tools for evaluation of treatment efficacy.

Response to treatment monitoring is done by a combination of clinical evaluation, imaging, mostly computed tomography (CT), and serum protein biomarkers such as CA19-9 [8, 9], and carcinoembryonic antigen (CEA) to a lesser extent. Clinical evaluations and CT imaging have significant drawbacks, such as being subjective and causing contrast nephrotoxicity, respectively. Hence, serum biomarkers are of great importance as a noninvasive and easy-to-use monitoring modality. Nevertheless, CA19-9 has many limitations. Most significantly, 5%–10% of patients will not express CA19-9 at all. Furthermore, there is significant racial and gender variation in CA19-9 expression, and there are benign conditions that cause CA19-9 elevation, including obstructive jaundice, which often complicates pancreatic cancer [10, 11]. Therefore, better noninvasive monitoring modalities are urgently needed.

The use of circulating tumor DNA (ctDNA) for monitoring of cancer has emerged in recent years. Several ctDNA markers are being used for treatment decisions, such as EGFR p.T790M for osimertinib treatment [12] and ARV7 for enzalutamide and abiraterone treatments [13]. Although ctDNA is mostly used as a "liquid biopsy" [14], none of these ctDNA markers are used serially for treatment follow-up. An effective treatment follow-up marker needs to be easy to use and specific and must closely correlate with disease response to treatment and progression.

We report herein serial monitoring of quantitative mutant *KRAS* ctDNA levels in metastatic pancreatic ductal adenocarcinoma (PDAC) patients, using a relatively inexpensive and readily available method for clinical use. We compare our results with known markers such as CA19-9.

Subjects, Materials, and Methods

Patients' Follow-Up and Plasma Samples

The study was approved by our local ethics committee. Seventeen patients with PDAC undergoing follow-up at the Division of Oncology at Rambam Health Care Campus in Haifa, Israel, were recruited for the study. Plasma samples were collected at each session at the time of venipuncture, prior to systemic oncological treatment. Follow-up visits included CA19-9 measurements and CT imaging, which were evaluated as part of the patients' routine follow-up. CtDNA *KRAS* mutant allele frequency was not revealed to the treating oncologist and did not affect patient management.

DNA Extraction from Plasma

Blood samples were drawn into EDTA tubes. Immediately following blood extraction, samples underwent centrifugation for 30 minutes at a speed of 6,000 × g to separate the plasma, in order to avoid lysis of blood cells and sample "contamination" with wild-type DNA that can hamper diagnosis of low-frequency mutations. DNA was extracted from the plasma using the MagMax Cell Free DNA isolation kit (Life Technologies, St. Austin, TX), according to manufacturer instructions; samples were incubated with magnetic beads for DNA binding and, following several washing and rebinding cycles, cell-free DNA was eluted. DNA concentration was measured using the Qubit Fluorometer (Invitrogen, Carlsbad, CA) and DNA quality as well as potential "contamination" with high-molecular-weight cellular wild-type DNA were determined using TapeStation 2200 (Genomic DNA Screen Tape; Agilent Technologies, Santa Clara, CA).

Mutation Analysis

Mutation analysis was performed using the Ion Torrent Personal Genome Machine (PGM) sequencer platform. ctDNA was polymerase chain reaction (PCR) amplified using *KRAS* exon 2 specific primers forward 5′-GGCCTGCTGAAAATGACTGAA-3′ and reverse 5′-GGTCCTGCACCAGTAATATGCA-3′. Each primer pair was supplemented with Ion-Torrent adapters P1 and A, to allow binding to the Ion Sphere Particles. Additionally, 20–30 different forward primers, each with a different barcode, were used for amplification to allow analysis of multiple samples in a single reaction. Amplicons were purified using the Qiagen PCR purification kit (Qiagen, Hilden, Germany) and then sequenced using an Ion 314 chip on the PGM for 65 cycles. We aimed for 10,000–20,000× coverage to allow identification of low-frequency mutations. Data from the PGM runs was initially processed using the Ion Torrent platform-specific pipeline software Torrent Suite v1.3.1 to generate sequence reads, trim adapter sequences, filter, and remove poor signal-profile reads. Generated sequence files were aligned to the genomic sequence of *KRAS* exon 2, and we determined the fraction of the mutation and the wild-type copies of the gene in each sample using the Integrative Genomic Viewer (IGV 2.3) free software [15, 16].

Determining the Analytical Sensitivity for Calling *KRAS* Mutations

The confounding factors for identifying low-frequency mutations with ultra-deep sequencing are the errors introduced during PCR and sequencing [17]. To define a substitution as a low-allele-frequency mutation rather than a sequencing artifact, we looked for a signal that is significantly higher than the background sequencing artifact "noise." To determine the noise level of our method, we calculated the distribution of non-hotspot nonreference substitutions in the bases sequenced from each case. Therefore, FASTQ files were aligned to the human genome using the Burrows-Wheeler Alignment Tool [18], and VCF files containing the reference and nonreference reads for each base were generated using SAMtools [19]. We assumed that non-hotspot alterations are most likely sequencing artifacts and not a true mutation and, therefore, these alterations were referred to as background "noise." Mutations higher than the 95th percentile background "noise" level should be defined as the detection limit of the method (a value higher than 95th percentile in the "hotspot" position has a chance of less than 5% to be an artifact; $p < .05$) [20]. However, there are six potential substitutions that lead to missense mutations for *KRAS* codon 12 mutations; therefore, to achieve an experiment-wide error rate of 0.05, an allele frequency

Table 1. Demographics and *KRAS* mutation status of the enrolled patients

Patient	Gender	Age	Survival from diagnosis (months)	Survival from enrollment (months)	*KRAS* mutation	Number of samples
1	F	73	44 (AWD)	27 (AWD)	Not detected	5
2	M	50	7	3	c.35G>A	2
3	F	61	9	6	c.35G>T	1
4	M	64	7	7	c.35G>A	4
5	M	40	19	17	Not detected	6
6	F	53	43 (AWD)	26 (AWD)	Not detected	8
7	M	61	7	6	Not detected	3
8	M	74	26 (AWD)	25 (AWD)	Not detected	3
9	M	59	31	6	Not detected	1
10	M	62	7	6	Not detected	2
11	M	58	8	7	c.35G>T	3
12	M	70	9	9	Not detected	3
13	M	73	18 (AWD)	18 (AWD)	Not detected	4
14	M	66	13	12	c.35G>T	3
15	M	66	56	9	Not detected	3
16	F	63	37.5	9	Not detected	2
17	F	69	14 (AWD)	13 (AWD)	Not detected	2

Abbreviations: AWD, alive with disease; F, female; M, male.

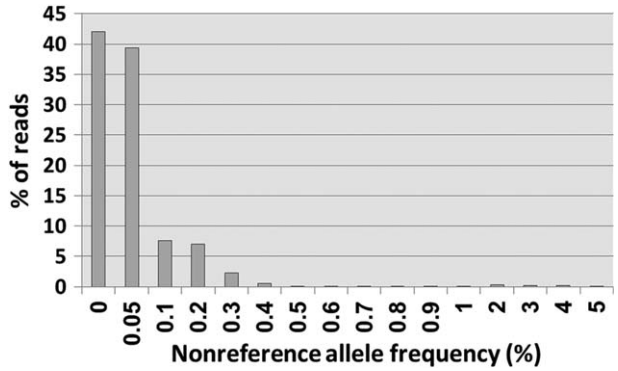

Figure 1. Analysis of sequencing artifact level. As detailed in Subjects, Materials, and Methods, sequencing results underwent bioinformatic analysis to determine allele frequency of non-hotspot nonreference sequences. Because we do not expect driver substitutions in non-hotspot positions, these were referred to as sequencing artifacts. As demonstrated in the figure, 42% of reads did not carry any nonreference substitutions, and in an additional 39%, the non-reference allele frequency was lower than 0.05%. The 99.17th percentile (equivalent to $p = .05$ after correction for multiple hypothesis) was 0.99%; hence, the limit of detection was set as 1%.

higher than the 99.17th percentile (equivalent to $p < .0083$) was defined as the limit of detection for the method.

Statistical Analysis

Univariate analysis of survival was done using the Kaplan-Meier product limits method [21], and survival curves were constructed. The log-rank test was used to compare between the *KRAS* mutation positive and negative groups. Correlation between the biomarker slope (ctDNA and CA19-9) and survival was evaluated using the Pearson's coefficient of correlations. For all statistical analyses, two-tailed p values of .05 or less were considered to be statistically significant.

Results

Mutant *KRAS* ctDNA Analysis

Seventeen patients, twelve males and five females, with metastatic PDAC were prospectively enrolled. All patients had biopsy-proven metastatic ductal carcinoma, except for one who was clinically diagnosed, based on relevant symptoms, imaging, and high CA19-9 levels. We collected 1–8 serial serum samples from all patients (median 3 samples). Same patient samples were collected at least 4 weeks apart. Table 1 displays patient characteristics.

Each plasma sample was collected in two 5-mL tubes. As detailed in Subjects, Materials, and Methods, true *KRAS* mutations were determined by comparing the frequency of nonreference reads in a hotspot position with the frequency of nonreference reads in a non-hotspot position. In the non-hotspot bases, the frequency of nonreference reads was $0.054\% \pm 0.133\%$ (Fig. 1). The 99.17th percentile was 0.99% and, therefore, *KRAS* codon 12 substitutions with allele frequency of higher than 1% were defined as mutations. Duplicate samples were in good agreement. There were two cases in which all the patient's samples were negative for *KRAS* mutations, except for one sample that had only one of the two duplicates positive. The discordance between duplicates in these two samples and the results of all other samples, before and after, in both patients led us to determine both samples as negative. Overall, using the methods described above, we detected plasma *KRAS* mutations in 5/17 patients (29.4%).

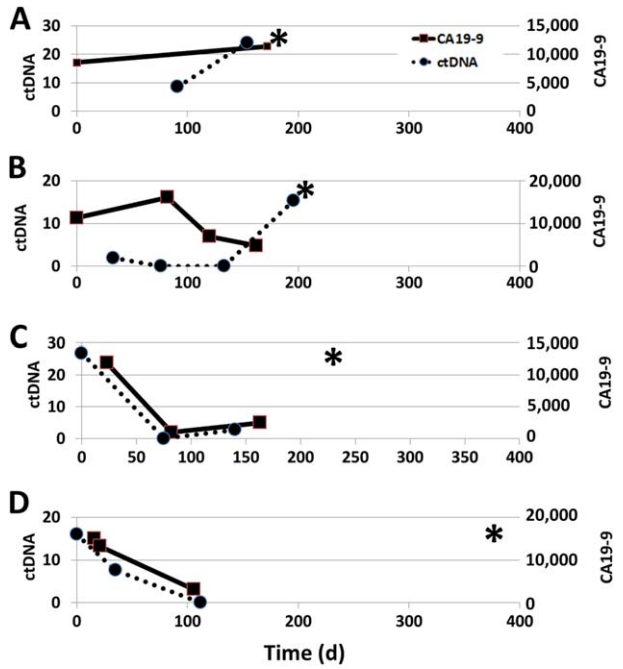

Figure 2. ctDNA and CA19-9 dynamics. Patients with pancreatic adenocarcinoma were followed, and blood samples were evaluated for CA19-9 levels and allele frequency of *KRAS* mutation. As demonstrated in the figure, in 3 of 4 cases (**A**, **C**, and **D**), CA19-9 and ctDNA showed similar dynamics in their values. In the case presented in **B**, ctDNA levels went up, suggesting progressive disease, whereas CA19-9 levels went down. Interestingly, the patient presented in B died shortly after the last measurement, indicating a progressive disease and suggesting that ctDNA might be a better predictor of disease progression than CA19-9. Additionally, rapid increase in ctDNA levels was associated with very short survival time (A and B). * represents time of death.

Abbreviations: ctDNA, circulating tumor DNA; d, days.

Mutant *KRAS* ctDNA Correlation with CA19-9, Imaging, and Clinical Outcome

For the five patients who had detectable serum mutated *KRAS* ctDNA, we followed CA19-9 and imaging to determine the prognostic value of this marker and the correlation with treatment response. These five patients were labeled in our study as numbers 2, 3, 4, 11, and 14 (Table 1). We show correlation of ctDNA levels, CA19-9, imaging, and clinical outcome for each patient.

Patient 2 had two serum samples and *KRAS* mutation c.35G>A was detected in both. Plasma levels were 8.65% at the first sample and 24.05% at the second (Fig. 2A). At the same time, CA19-9 levels were raised, and CT imaging showed disease progression, suggesting good correlation between all three monitoring modalities.

Patient 3 had one sample drawn and, therefore, was unavailable for correlation analysis with other monitoring modalities.

Patient 4 had four samples analyzed (Fig. 2B). There was no correlation between ctDNA, CA19-9, and imaging. At first, while the patient was receiving chemotherapy, ctDNA levels were below the detection threshold and CA19-9 levels were diminishing, but imaging showed disease progression. Then, as seen in Figure 2B, ctDNA levels rose and CA19-9 levels diminished further but, shortly afterward, the patient died due to his disease. Therefore, in this specific patient, ctDNA levels were more predictive of clinical outcome than CA19-9.

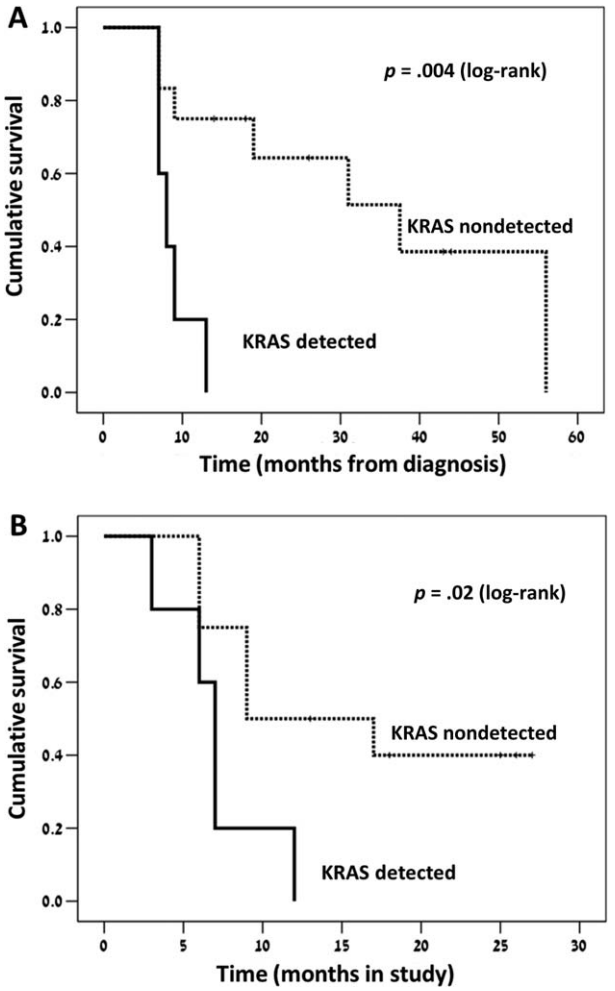

Figure 3. Mutant *KRAS* circulating tumor DNA levels and survival. Survival analysis showed that the absence of detectable *KRAS* mutation in the plasma was associated with statistically significant survival benefit. This was true both for survival from diagnosis **(A)** and for survival from enrollment in the study **(B)**.

Patient 11 (Fig. 2C) responded at first to treatment with a significant reduction of both CA19-9 (11,900 to 969) and ctDNA levels (26.85 to undetected) and a partial response on imaging, then showed disease progression by CA19-9 (969 to 2,491) and by ctDNA (undetected to 2.75) and tumor progression on imaging. Therefore, this patient had good correlation between ctDNA, CA19-9 expression, and imaging.

Patient 14 (Fig. 2D) also showed correlation between CA19-9 and ctDNA levels, both going down. However, no imaging was performed to evaluate his disease during, soon before, or soon after ctDNA samples were drawn. Clinically, the patient survived 274 days after his last sample, suggesting that the diminishing ctDNA levels correlated well with his relatively long survival afterward.

Overall, our results suggest good correlation between CA19-9 levels and ctDNA levels.

Mutant *KRAS* ctDNA as a Prognostic Marker in Metastatic Pancreatic Cancer Patients

We tested whether there was correlation between ctDNA mutant *KRAS* detection and prognosis. Of note, 5 of our 17 patients remained alive at the end of study (14–44 months

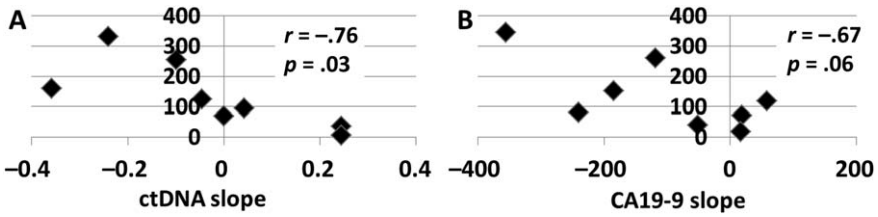

Figure 4. The association between ctDNA and CA19-9 dynamics and survival. We calculated the slope of ctDNA and CA19-9 between every two consequent measures (ΔctDNA/Δtime and ΔCA19-9/Δtime. ctDNA slope showed statistically significant association with survival time, with higher slopes being associated with shorter survival time **(A)**. CA19-9 slope showed only a trend toward association with survival **(B)**. This finding suggests that ctDNA dynamics might be a better predictor of survival in pancreatic cancer than CA19-9 dynamics.
Abbreviation: ctDNA, circulating tumor DNA.

from diagnosis) and none had a detectable serum *KRAS* mutation. This prompted us to test whether detection of mutant *KRAS* could be a poor prognostic marker for metastatic pancreatic carcinoma patients. To answer this question, we performed a Kaplan-Meier analysis [21] of all patients in the study, stratified by detectable/nondetectable mutant *KRAS* ctDNA. Our analysis shows that undetectable mutant *KRAS* in the plasma was associated with favorable prognosis (8 vs. 37.5 months from diagnosis, $p < .004$; Fig. 3).

Next, we wanted to test whether the dynamics of ctDNA levels correlated with patients' survival. Toward this aim, we calculated the slope of ctDNA levels, defined as the change in ctDNA levels between each pair of consecutive samples divided by the difference between sample times. Each slope value was paired with the time from the second sample to death. Using Pearson's coefficient of correlation, we found significant negative correlation between the ctDNA slopes and survival times ($r = -0.76$, $p = .03$; Fig. 4A). Hence, a deep fall in ctDNA expression over a short period of time correlates with longer survival time, whereas a fast and marked rise in ctDNA level correlates with poor prognosis. In order to compare the prognostic value of ctDNA slope with the standard serum biomarker CA19-9, we calculated the correlation between CA19-9 slope and prognosis using the same algorithm. Our results showed no significant correlation between the change in CA19-9 and survival ($r = -0.67$, $p = .06$; Fig. 4B).

Discussion

Detection and analysis of circulating tumor DNA for somatic driver mutations is an evolving field, clinically and academically. A search for the term "circulating tumor DNA" in PubMed showed a continuous rise in the number of publications, with 301, 418, and 507 articles published in 2014, 2015, and 2016, respectively. Moreover, ctDNA analyses for specific mutations are now part of the clinical routine for a number of tumors [22]. Single-mutation ctDNA analysis has a specifically high clinical utility in tumors with common driver mutations. Sixty-three to ninety-three percent of PDAC harbor *KRAS* mutations [23], making it an excellent candidate marker for monitoring metastatic PDAC patients.

In the present study, we identified *KRAS* mutations in the plasma of five (29.4%) of our patients. Previous studies reported similar results, with identification of *KRAS* mutations in 8/31 patients studied [24]. One potential explanation for the relatively low percentage of *KRAS* positive ctDNA is that some of the tumors in our cohort did not carry a *KRAS* mutation. Sequencing a larger gene panel would increase the chances of identifying a tumor-specific ctDNA marker [14]. Another reason for the relatively low frequency of cases with detectable ctDNA mutant *KRAS* could be the limit of detection of our assay. The amount of ctDNA released from the tumor changes with time and with alterations in tumor turnover [25]. In some tumors, the amount of ctDNA can be less than 10 mutant copies per 5 mL of plasma [26]. In these cases, more sensitive testing modalities are needed [17, 27, 28].

Interestingly, the cases that were ctDNA negative in our cohort had a significantly better survival, suggesting that ctDNA could correlate with disease burden or activity. Similar to our findings, previous studies have also highlighted the association between the presence of detectable *KRAS* mutation in plasma and poor survival in patients with resectable [29] and advanced [24] pancreatic cancer.

Another evolving promising form of liquid biopsies is exosome isolation. Exosomes are cell-derived vesicles that can be isolated from the blood. Tumor exosomes represent an excellent candidate as a liquid biopsy because they contain high-quality DNA and are relatively easy to isolate [30] through the use of exosomal surface markers such as glypican1 [31]. Whole exosome sequencing is enriched with tumor DNA and can detect 90%–95% of target regions [32], even without enrichment for tumor-related exosomes. For pancreatic cancer specifically, tumor DNA with *KRAS* and *TP53* mutations could be detected in exosomes [33].

Currently, follow-up of patients with PDAC is based on clinical evaluation, CA19-9 levels, and imaging studies. In a study of 944 patients, postsurgical CA19-9 normalization was an independent predictor of good overall survival and progression-free survival [34]. Additionally, CA19-9 levels before surgery have been inversely associated with survival [35]. Nevertheless, analysis of CA19-9 would yield a false-negative result in a fraction of patients not expressing the Lewis antigen [10]. Furthermore, patients with several non-malignancy-related clinical conditions, such as obstructive jaundice and diabetes [10, 36], express falsely elevated CA19-9 levels. Therefore, the need to include additional protein markers to improve the specificity of CA19-9 in PDAC was suggested in several studies [37].

In this study, on a case-by-case basis, we found good correlation between *KRAS* mutant allele frequency and the serological (CA19-9) and imaging study results. To the best of our knowledge, this is the first study looking into the association between ctDNA levels and protein markers, such as CA19-9, in pancreatic cancer. We found that the dynamics of mutant *KRAS*

ctDNA level changes were in closer association with survival than CA19-9 dynamics. In one case, a rapid increase in mutant *KRAS* ctDNA levels was associated with a very short survival time, while CA19-9 continued to go down. The potentially superior predictive value of ctDNA levels, suggested by our observations, can be explained by the different half-life of protein markers and DNA in the plasma. The average half-life of CA19-9 was 0.5 days in the first compartment and 4.3 days in the second compartment [38], whereas ctDNA has a much shorter half-life of approximately 2 hours [39], making it a more suitable marker for short-term dynamics. However, it is important to note that the small number of patients used in our analysis and the relatively low frequency of ctDNA *KRAS* mutation detection are a significant limitation of our study. These results should be further validated in larger studies, before clinical practice can change, to include mutant *KRAS* ctDNA as a marker guiding treatment decisions.

Conclusion

We found that ctDNA detection in PDAC and its expression level dynamics can be used for prognosis determination and disease monitoring, respectively. The relative ease of analysis, modest costs, and the clues we found for superiority compared with the currently used protein markers suggest that ctDNA could be an excellent clinically applicable biomarker for PDAC. Future studies should further validate its role and evaluate the potential application of other types of liquid biopsies, such as exosomal DNA [40].

Author Contributions

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Data analysis and interpretation: Ruth Perets, Orli Greenberg, Talia Shentzer, Tova Bick, Shada Sarji, Dov Hershkovitz

Manuscript writing: Ruth Perets, Orli Greenberg, Dov Hershkovitz

Final approval of manuscript: Ruth Perets, Orli Greenberg, Talia Shentzer, Valeria Semenisty, Ron Epelbaum, Tova Bick, Shada Sarji, Ofer Ben-Izhak, Edmond Sabo, Dov Hershkovitz

Disclosures

The authors indicated no financial relationships.

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For Further Reading:

Karen-Lise G. Spindler, Anders K. Boysen, Niels Pallisgård et al. Cell-Free DNA in Metastatic Colorectal Cancer: A Systematic Review and Meta-Analysis. ***The Oncologist*** 2017;22:1049–1055.

Implications for Practice:

Reliable prognostic markers could help to guide patients and treating physicians regarding the relevance and choice of systemic therapy. Small fragments of circulating cell-free DNA (cfDNA) can be measured in a simple blood sample. This report presents the first meta-analysis of the prognostic value of total cfDNA measurement in patients with metastatic colorectal cancer. Data from 1,076 patients confirmed that patients with the lowest pre-treatment levels of cfDNA had a significantly higher chance of longer survival than those with higher levels. Cell-free DNA analysis can also be used for detection of tumor-specific mutations, and hold potential as a valuable tool in colorectal cancer treatment.