



Combined circulating tumor DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers

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Contributed by Bert Vogelstein, May 4, 2017 (sent for review April 4, 2017; reviewed by Daniel A. Haber and Lance Liotta)

The earlier diagnosis of cancer is one of the keys to reducing cancer deaths in the future. Here we describe our efforts to develop a noninvasive blood test for the detection of pancreatic ductal adenocarcinoma. We combined blood tests for *KRAS* gene mutations with carefully thresholded protein biomarkers to determine whether the combination of these markers was superior to any single marker. The cohort tested included 221 patients with resectable pancreatic ductal adenocarcinomas and 182 control patients without known cancer. *KRAS* mutations were detected in the plasma of 66 patients (30%), and every mutation found in the plasma was identical to that subsequently found in the patient's primary tumor (100% concordance). The use of *KRAS* in conjunction with four thresholded protein biomarkers increased the sensitivity to 64%. Only one of the 182 plasma samples from the control cohort was positive for any of the DNA or protein biomarkers (99.5% specificity). This combinatorial approach may prove useful for the earlier detection of many cancer types.

early cancer detection | liquid biopsy | circulating tumor DNA | protein biomarkers | pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC, hereafter “pancreatic cancer”) is the third leading cause of cancer death and is predicted to become the second most common cause in the United States by 2030 (1). Pancreatic cancer is notoriously lethal, with fewer than 9% of patients surviving 5 y after diagnosis (2). The poor prognosis of patients with pancreatic cancer is in part due to the fact that 80–85% of patients are diagnosed at advanced stages, when either tumor invasion into the surrounding major vessels or distant metastases are evident upon radiologic studies (3). At this late point in the disease, pancreatic cancer is not amenable to surgical resection, and the 3-y survival rate is <5%. In contrast, a 5-y survival of almost 60% is reported for very small, localized tumors; among resectable cancers, the smaller the tumor, the better the prognosis (4–8).

Pancreatic cancer is not different from other cancers with respect to its strong correlation between tumor stage and prognosis (4). Very few patients with cancers of the lung, colon, esophagus, or stomach who have distant metastasis at the time of diagnosis survive for more than 5 y (9). The size of cancers is also important in a general sense, in that smaller tumors have less often metastasized than larger tumors at the time of diagnosis and are therefore more likely to be curable by surgery alone. Even when cancers have metastasized to distant sites, a smaller burden of disease is much more easily managed than bulky lesions (10). Thus, adjuvant chemotherapeutic agents administered to patients

Author contributions: J.D.C., A.A.J., F.W., Y.W., N.P., K.W.K., B.V., and A.M.L. designed research; J.D.C., A.A.J., C. Thoburn, Y.W., L.L., J.P., L.D., J.S., N.S., M.P., N.P., K.W.K., B.V., and A.M.L. performed research; J.D.C., A.A.J., J.T., P.G., C.M.S., M.T.Y.-S., P.J.A., M.S., R.E.B., A.D.S., G.M.P., S.-M.H., S.C.K., M.F., C.D., M.J.W., N.A., J.H., M.A.M., A.M., S.M.H., M.D.M., Y.W., L.L., M.G.G., R.H.H., C.L.W., C. Tomasetti, N.P., K.W.K., B.V., and A.M.L. contributed new reagents/analytic tools; C. Tomasetti designed the algorithm for the sequencing data analysis; J.D.C., A.A.J., C. Thoburn, F.W., Y.W., L.L., A.P.K., C. Tomasetti, N.P., K.W.K., B.V., and A.M.L. analyzed data; and J.D.C., A.A.J., F.W., N.P., K.W.K., B.V., and A.M.L. wrote the paper.

Reviewers: D.A.H., Massachusetts General Hospital; and L.L., George Mason University.

Conflict of interest statement: C.M.S. and M.T.Y.-S. are founders and coowners of B9, Inc. and have no conflicts of interest with respect to the new technology described in this article. N.P., K.W.K., and B.V. are founders of Personal Genome Diagnostics, Inc. and PapGene, Inc. K.W.K. and B.V. are members of the Scientific Advisory Board of Sysmex-Inostics and Morphotek. B.V. is also a member of the Scientific Advisory Board of Exelixis GP. These companies and others have licensed technologies from Johns Hopkins, including those related to early diagnostics. N.P., K.W.K., and B.V. are the inventors of some of these technologies and receive equity or royalties from their licenses. The terms of these arrangements are being managed by the university in accordance with its conflict of interest policies. N.P., K.W.K., and B.V. have no conflicts of interest with respect to the new technology described in this article, as defined by the Johns Hopkins University policy on conflict of interest.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. [EGAS000011002444](https://www.ncbi.nlm.nih.gov/nuclseq/EGAS000011002444)).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1704961114/-DCSupplemental.

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Significance

Few patients with pancreatic cancer survive longer than 5 y, in part because most patients are identified only after their disease has progressed to an advanced stage. In this study, we show how combining mutations in circulating tumor DNA (ctDNA) with protein markers can result in a screening test with improved sensitivity while retaining specificity. The combination of the ctDNA and protein markers was superior to any single marker. Moreover, the combination detected nearly two-thirds of pancreatic cancers that had no evidence of distant metastasis at the time of surgical resection. The strategy may represent an approach to detect cancers of many types at an earlier stage.

with micrometastases stemming from a colorectal cancer can be curative in nearly 50% of cases (11–13). The same chemotherapeutic agents delivered to patients with metastatic lesions that are radiologically visible produce virtually no cures (14).

It is therefore evident that the earlier detection of cancers is one key to reducing deaths from these diseases, including pancreatic cancer. In addition to offering the possibility of surgical resection, newly developed adjuvant chemotherapeutic and emerging immunotherapy regimens will undoubtedly prove more efficacious in patients with minimal disease beyond that which is curable surgically (15). Biomarkers in the circulation provide one of the best ways, in principle, to detect cancers at an earlier stage. Historically, the type of biomarkers used to monitor cancers were proteins (16) and included carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and cancer antigen 125 (CA125). These biomarkers have proven useful for following patients with known disease, but none has been approved for screening purposes, in part because of their low sensitivity or specificity (17–19). More recently, mutant DNA has been explored as a biomarker. The concept underlying this approach, often called “liquid biopsies,” is that cancer cells, like normal self-renewing cells, turn over frequently. DNA released from the dying cells can escape into bodily fluids such as urine, stool, and plasma (20–30). An advantage of using mutant DNA in the circulation as a biomarker is its exquisite specificity. Every cell within a cancer has a core set of somatic mutations in driver genes that are responsible for their clonal growth (31). In contrast, normal cells do not clonally expand during adulthood, and the fraction of normal cells that have any specific somatic mutation is extremely low.

Most studies of circulating tumor DNA (ctDNA) have focused on following patients with cancer rather than on evaluating its use in screening settings. Available data indicate that ctDNA is elevated in >85% of patients with advanced forms of many cancer types (22, 24). However, it has been shown that a considerably smaller fraction of patients with earlier stages of cancer have detectable levels of ctDNA in their plasma (22, 24). In the current study, we determined whether ctDNA and protein biomarkers can

be combined to increase the sensitivity of detection of resectable pancreatic cancer under conditions that preserve high specificity.

Results

Characteristics of Patients with PDAC and Presumed Healthy Controls.

Two hundred and twenty-one patients with surgically resectable pancreatic cancer were evaluated in this study. The histopathological and clinical characteristics of these patients are summarized in Table S1. A total of 182 individuals of similar age with no known history of cancer, autoimmune disease, or chronic kidney disease acted as the healthy control cohort.

Twenty percent of the patients had no symptoms typically associated with pancreatic cancer. The size of the primary tumors at presentation ranged from 0.6 cm to 13 cm, with a median size of 3.0 cm. The most common stage at presentation was American Joint Commission on Cancer (AJCC) stage IIB, accounting for 77% of patients, with the remaining patients harboring stage IA (5%), stage IB (8%), or stage IIA (10%) disease (Table S1). Patient survival correlated with stage, as graphically depicted in Fig. S1 and as expected from prior clinical studies (32).

A PCR-Based Assay to Identify Tumor-Specific *KRAS* Mutations in Plasma Samples.

We designed a PCR-based assay that could simultaneously assess the two codons (codons 12 and 61) of the *KRAS* gene that are most frequently mutated in PDAC as well as surrounding codons. The assay used a sensitive technology called the “Safe-Sequencing System” (Safe-SeqS) (33). Safe-SeqS incorporates molecular barcodes that uniquely label each template molecule, thereby drastically minimizing the errors that routinely occur in massively parallel sequencing. This approach can identify one mutant template among as many as 10,000 normal templates. Using this technology, we identified *KRAS* mutations in the plasma of 66 of the 221 (30%; 95% CI 24–36%) pancreatic cancer cases (Table 1, Table S2, and Dataset S1). Sixty-two (94%) and four (6%) of the mutations were at codons 12 and 61, respectively, with G > T transversions most commonly observed (Dataset S1). Mutations were found more frequently in stage II patients than in stage I patients (Fig. 1A, Table 1, Table S2, and Dataset S1). Additionally, while the mutant allele frequency did not correlate with tumor size (Fig. S2A and Dataset S1), mutations were found more frequently in larger tumors than in smaller tumors (Fig. 1B, Table 2, Table S2, and Dataset S1).

The number of mutant templates in the plasma could be calculated from the mutant allele fraction and the concentration of DNA in each plasma sample (Dataset S1). This number was often very low, with 25 (38%) of the patients with detectable *KRAS* mutations having fewer than two mutant templates per milliliter of plasma. The average number of mutant templates per milliliter of plasma was 5.3 (Dataset S1). These results emphasize that extremely sensitive techniques are required to effectively detect the mutations in early-stage pancreatic cancer patients. *KRAS* mutations were observed in only one of the 182 individuals in the presumed healthy cohort, a 69-y-old male with no known cancer.

Table 1. Proportion of samples stratified by AJCC stage detected with each individual assay and all combinations thereof

Assay type	% samples detected (95% confidence interval)				
	Stage IA, 12 cases	Stage IB, 17 cases	Stage IIA, 22 cases	Stage IIB, 170 cases	Stage I and II, 221 cases
ctDNA	25 (5–57)	0 (0–20)	18 (5–40)	35 (28–42)	30 (24–36)
CA19-9	17 (2–48)	41 (18–67)	36 (17–59)	54 (46–62)	49 (43–56)
CEA + HGF + OPN	25 (5–57)	6 (0–29)	14 (3–35)	19 (14–26)	18 (13–24)
ctDNA + CA19-9	33 (10–65)	41 (18–67)	50 (28–72)	65 (58–72)	60 (53–67)
ctDNA + CEA + HGF + OPN	33 (10–65)	6 (0–29)	32 (14–55)	47 (39–55)	42 (35–48)
CA19-9 + CEA + HGF + OPN	25 (5–57)	47 (23–72)	36 (17–59)	59 (52–67)	54 (47–61)
Combination assay	33 (10–65)	47 (23–72)	50 (28–72)	69 (62–76)	64 (57–70)

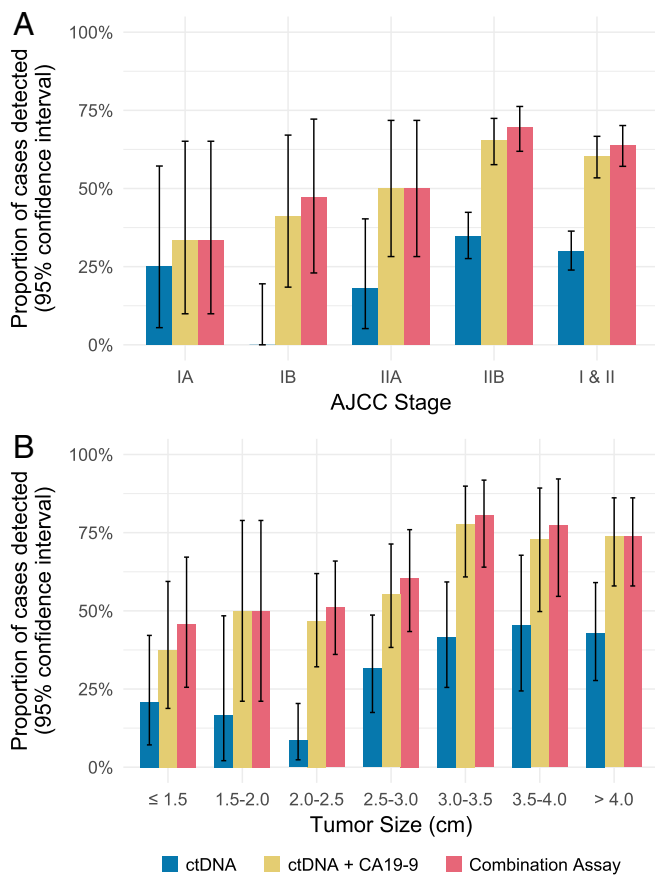


Fig. 1. Combining ctDNA *KRAS* mutations with protein biomarkers increases sensitivity for early detection of PDAC. (A) Sensitivities of ctDNA *KRAS* mutations alone, ctDNA *KRAS* mutations plus CA19-9, and ctDNA *KRAS* mutations with CA19-9 and other proteins (combination assay) with respect to AJCC stage. (B) Sensitivities of ctDNA *KRAS* mutations alone, ctDNA *KRAS* mutations plus CA19-9, and ctDNA *KRAS* mutations with CA19-9 and other proteins (combination assay) with respect to tumor size. Error bars represent 95% CIs.

The basis for the liquid biopsy concept is that the mutant DNA templates identified in the circulation are derived from cancers. It was therefore important to determine whether the *KRAS* mutations identified in these patients' plasma samples were also present in their primary carcinomas. We were able to obtain primary carcinomas from 50 of the 66 patients with detectable *KRAS* mutations in their plasma. In all 50 cases, the mutation found in the plasma was identical to that found in the primary carcinoma, providing another, orthogonal measure of specificity.

Simultaneous Assessment of CA19-9 and *KRAS* Mutations in Plasma.

We sought to determine whether a combination of the *KRAS* ctDNA test with CA19-9, the best-known PDAC biomarker (17, 34), would result in improved sensitivity compared with the *KRAS* ctDNA test alone. Recent studies have shown that CA19-9 can be elevated in patients with pancreatic cancer 2 y before diagnosis (35). However, CA19-9 elevations have also been observed in nonmalignant conditions, and 5% of the population cannot produce the CA19-9 antigen due to germline genetic variation, limiting its use for screening purposes (17). However, we reasoned that CA19-9 might prove useful as a screening biomarker if the threshold for scoring a result as positive was sufficiently high. We chose a threshold of 100 U/mL based on prior data that this level is not found among healthy individuals who do not have a clinical history of pancreaticobiliary disease (36).

Using this predefined high threshold, CA19-9 was detected in 109 of the 221 (49%; 95% CI 43–56%) patients with pancreatic cancer and in none of the 182 healthy controls, confirming its specificity when used in this way (Table 1, Table S3, and Datasets S1 and S2). As expected, the number of patients with detectable CA19-9 levels increased with stage and tumor size (Fig. 1, Tables 1 and 2, Table S3, and Dataset S1). The most important question addressed in the current study was whether these two biomarkers—*KRAS* mutations and a positive CA19-9 score—were independent indicators of the presence of disease. We found that the overlap was only partial, as indicated in the Venn diagram in Fig. 24. Although 42 patients (19%) had elevated CA19-9 levels as well as detectable *KRAS* mutations in their plasma, 91 additional patients had either mutations in *KRAS* or elevated CA19-9 but not both (Fig. 24). Thus, the combined sensitivity of these analyses was 60% (95% CI 53–67%), higher than the sensitivity of either alone (Fig. 1 and Table 1). Importantly, the two assays could be combined without substantially increasing the false-positive rate because each was extremely specific at the thresholds used.

Further Increasing Sensitivity by Inclusion of Other Protein Biomarkers.

Encouraged by the results described above, we sought to further increase sensitivity by combining ctDNA *KRAS* mutations and CA19-9 with other protein biomarkers. In a pilot study on a small number of pancreatic cancer samples independent from those studied here, we evaluated the potential utility of other proteins that had been found to be elevated in cancer, including alpha-fetoprotein (AFP), CA15-3, leptin, IL-6, CEA, CA-125, IL-8, sFas, prolactin, osteopontin (OPN), basic FGF2, hepatocyte growth factor (HGF), cytokeratin-19 fragment (CYFRA 21-1), human epididymis protein 4 (HE4), TGF- α , growth/differentiation factor 15 (GDF15), dickkopf-related protein 1 (DKK1), neuron-specific enolase (NSE), osteoprotegerin (OPG), TIMP metalloproteinase inhibitor 1 (TIMP-1), TIMP metalloproteinase inhibitor 2 (TIMP-2), mesothelin, midkine, kallikrein-6, CD44, AXL receptor tyrosine kinase, soluble human epidermal growth factor receptor 2 (sHER2), soluble epidermal growth factor receptor (sEGFR), soluble urokinase-type plasminogen activator receptor (suPAR), and soluble platelet endothelial cell adhesion molecule 1 (sPECAM-1) (37). Of these 29 protein biomarkers, five—CEA (38), HGF (39), midkine (40), OPN (41), and prolactin (42)—appeared to be most promising.

When the levels of these five markers were evaluated in plasmas from our 221-patient pancreatic cancer cohort, we observed an association between the plasma concentrations of prolactin and midkine and sample collection site ($P < 0.01$, χ^2 test, $df = 5$), suggesting that blood collection conditions might have elevated the levels of these two markers. There was no significant correlation between CA19-9, CEA, HGF, or OPN levels and collection sites, nor was there any correlation between the presence of *KRAS* ctDNA mutations and collection site. Upon further investigation, we noted that the levels of prolactin and midkine were significantly elevated in samples that were collected after the administration of anesthesia but before surgical excision (Fig. S3). The results on prolactin were consistent with previous studies showing that anesthetics elevate the levels of this protein (43). To ensure that anesthesia did not affect the levels of the other protein biomarkers described above, we collected paired plasma samples before and immediately after the administration of anesthesia in 29 new patients. The only proteins found to be elevated by anesthesia were prolactin and midkine ($P < 0.05$, Wilcoxon signed-rank test) (Fig. S4), in perfect accord with the correlation between collection site and protein levels noted above. We therefore excluded prolactin and midkine from further analysis.

Unlike CA19-9, no predefined threshold exists for the use of CEA, HGF, or OPN as markers for pancreatic cancer. As a result, we determined appropriate thresholds in an independent set of 273 plasma samples from healthy controls. To be conservative,

Table 2. Proportion of samples stratified by tumor size detected with each individual assay and all combinations thereof

Assay type	% samples detected (95% confidence interval)						
	≤ 1.5 cm, 24 cases	1.5–2.0 cm, 12 cases	2.0–2.5 cm, 47 cases	2.5–3.0 cm, 38 cases	3.0–3.5 cm, 36 cases	3.5–4.0 cm, 22 cases	>4.0 cm, 42 cases
ctDNA	21 (7–42)	17 (2–48)	9 (2–20)	32 (18–49)	42 (26–59)	45 (24–68)	43 (28–59)
CA19-9	25 (10–47)	33 (10–65)	43 (28–58)	45 (29–62)	58 (41–74)	59 (36–79)	67 (50–80)
CEA + HGF + OPN	25 (10–47)	8 (0–38)	17 (8–31)	21 (10–37)	8 (2–22)	18 (5–40)	24 (12–39)
ctDNA + CA19-9	38 (19–59)	50 (21–79)	47 (32–62)	55 (38–71)	78 (61–90)	73 (50–89)	74 (58–86)
ctDNA + CEA + HGF + OPN	38 (19–59)	25 (5–57)	26 (14–40)	47 (31–64)	47 (30–65)	55 (32–76)	50 (34–66)
CA19-9 + CEA + HGF + OPN	38 (19–59)	33 (10–65)	47 (32–62)	53 (36–69)	64 (46–79)	64 (41–83)	67 (50–80)
Combination assay	46 (26–67)	50 (21–79)	51 (36–66)	61 (43–76)	81 (64–92)	77 (55–92)	74 (58–86)

we chose thresholds for each protein that were 10% higher than the maximum values observed in any of the 273 normal plasma samples. Notably, when these thresholds were applied to the independent test set of 182 plasma samples, all three protein markers maintained 100% specificity (Dataset S2). The sensitivity of each of these three markers was less than that obtained with *KRAS* mutations or CA19-9 when each marker was used alone, but their levels were less dependent on stage and size than *KRAS* mutations or CA19-9 (Table 1, Fig. S2, Tables S4–S6, and Dataset S1). In combination with *KRAS* mutations and CA19-9 assays, this five-member biomarker panel (“combination assay”) detected 141 (64%; 95% CI 57–70%) of the 221 resectable cancers (Figs. 1A and 2A, Table 1, Fig. S5, Table S7, and Dataset S1).

Some of the patients detectable by the combination assay were of particular note. Forty-five (20%) of the patients in this study had no symptoms classically associated with pancreatic cancer (Table S1 and Dataset S1). The combination assay identified 27 (60%) of these individuals, of whom 19 (70%) had no evidence of recurrence with a median follow-up of 12 mo (range 3–16 mo) (Table S7 and Dataset S1). Of the 29 patients with the earliest stages of disease recognized by the AJCC (stages IA and IB), 12 (41%) were detectable using the combination assay (Fig. 1A), seven of whom (58%) had no evidence of recurrence at the study termination with a median follow-up of 19 mo (range 2–25 mo).

Another notable but sobering result from our study was that patients with poorer survival were more likely to have a positive test. Of the entire 221 pancreatic cancer patients studied, 122

(56%) patients were alive at the termination of the study, with a median follow-up of 13 mo (7–21 mo). We found that the combination assay provided prognostic value that was independent of conventional clinical and histopathologic features. In particular, multivariate analyses showed that the independent predictors of overall survival were combination assay status [hazards ratio (HR) = 1.76, 95% CI 1.10–2.84, $P = 0.018$], increasing age (HR = 1.04, 95% CI 1.02–1.06, $P = 0.001$), grade of differentiation (poorly differentiated, HR = 1.72, 95% CI 1.11–2.66, $P = 0.015$), lymphovascular invasion (present, HR = 1.81, 95% CI 1.06–3.09, $P = 0.028$), nodal disease (present, HR = 2.35, 95% CI 1.20–4.61, $P = 0.013$), and margin status (HR = 1.59, 95% CI 1.01–2.55, $P = 0.050$) (Fig. S6).

Inclusion of *TP53* in a Multiplex Assay. While nearly all pancreatic cancers harbor mutations within *KRAS*, a large fraction (~75%) also contains mutations in *TP53* (44–46). Furthermore, *TP53* is the mostly commonly mutated gene in cancer (31), making it an attractive target for ctDNA detection in future studies involving other tumor types. We wished to determine whether the mutant allele frequencies (MAF) of *TP53* in the plasma correlated with those of *KRAS* and also whether a mutant *TP53* assay in plasma might add to the sensitivity of the mutant *KRAS* assay. For this purpose, we evaluated the 152 carcinomas for which matched tumor and plasma samples were available. We first searched for mutations at one of the “hotspots” identified in previous genome-wide studies of PDAC (44). A total of 64 (42%) carcinomas contained a *TP53* mutation at one of these positions. We then determined whether these same mutations could be identified in the plasma of these 64 patients, using Safe-SeqS-based assays similar to that described above for *KRAS* but using primers specific for particular *TP53* mutations.

We identified *TP53* mutations in 13 (20%) of the 64 plasma samples (Table S8). Two observations were of interest. First, 12 of the 13 plasma samples containing a detectable *TP53* mutation also contained a detectable *KRAS* mutation. Thus, *TP53* mutation assays did not substantially increase sensitivity for pancreatic cancer detection, as expected from the high prevalence of *KRAS* mutations noted above. Second, there was a strong correlation between the MAF of *TP53* and *KRAS* mutations in the plasma of the 12 patients whose plasma contained detectable amounts of both mutations (Pearson’s $r = 0.885$) (Fig. 2B). This provides yet another validation of the reliability of the ctDNA assay and its quantitative nature.

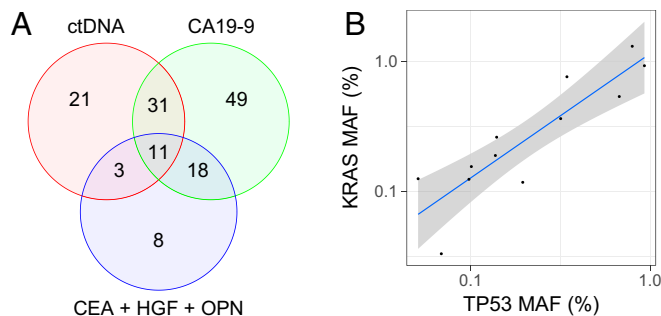


Fig. 2. (A) Combining ctDNA and protein markers increases sensitivity because a large proportion of patients are detected by only one marker. The Venn diagram shows the number of patients detected by ctDNA *KRAS* mutations (red circle), CA19-9 (green circle), the three other protein biomarkers (blue circle), and by combinations thereof (overlapping regions). Eighty patients (36% of the total) were not detectable by any of the three markers. (B) MAF of *KRAS* and *TP53* mutations are strongly correlated (Pearson’s $r = 0.885$) in the plasma of the 12 patients whose plasma contained detectable amounts of both mutations, providing validation of the reliability of the ctDNA assay and its quantitative nature. The shaded region represents the 95% CI.

Discussion

The major conclusion of this study is that assays for genetic alterations can be combined with assays for elevated proteins to increase the sensitivity of a blood test for low-stage pancreatic cancers. We were able to detect 64% of these cancers through this combination test, including some patients with a favorable prognosis. One of the critical design features of our study was that we included only patients with resectable pancreatic cancers and excluded all patients with advanced disease (i.e., stage III or

IV). Although this exclusion reduces the sensitivity that could be achieved by evaluating all pancreatic cancer patients, regardless of stage, the resectable cases are the most important group with respect to evaluating a screening technology.

Whether combining ctDNA and protein markers could increase sensitivity over either alone was not known. In fact, it was conceivable that the patients with detectable circulating protein markers would largely overlap those releasing DNA into the circulation. This was of particular concern for early-stage cancer patients, because both ctDNA and protein-based markers are known to be considerably higher in patients with advanced cancers than in those with earlier-stage cancers (17, 19, 22).

Another important aspect of our study is that we achieved very high specificity (99.5%: 95% CI 97–100%), observing only one false positive among 182 healthy individuals of average age 64 y. Given the relative infrequency of cancer in the general population, the specificity of any potentially useful blood-based screening test for pancreatic cancer has to be high, preferably >99%. Otherwise, the number of false positives will greatly exceed the number of true positives (i.e., have suboptimal positive predictive value) (47). This stringency for screening tests is not required for tests to monitor disease in patients with known cancer. For monitoring, specificity can be relaxed somewhat in the interest of obtaining higher sensitivity. We achieved high specificity in two ways. First, we used ctDNA as one of the components of the test. *KRAS* mutations are exquisitely specific for neoplasia, and their specificity is limited by technical rather than biological factors. The incorporation of molecular barcoding into our assays (Safe-SeqS) minimizes the false-positive results from sequencing that are the major technical issues confronting any ctDNA-based assays. *KRAS* mutations are particularly suitable for early-detection strategies because they are rarely found in clones arising during age-associated clonal hematopoiesis. Such clones, which may represent early forms of myelodysplasia, are a potential source of false-positive ctDNA assays. The vast majority of such mutations occur within nine genes (*DNMT3A*, *TET2*, *JAK2*, *ASXL1*, *TP53*, *GNAS*, *PPM1D*, *BCORL1*, and *SF3B1*) (48–50), posing challenges for the use of these genes as biomarkers in ctDNA-based assays. Second, we used high thresholds for scoring the protein markers as positive. These thresholds were based on prior studies in the literature or on an independent set of controls, allowing us to avoid positive scores in the vast majority of healthy patients (36). We could afford to use these high thresholds without an overall reduction in sensitivity because the ctDNA assay added sensitivity on its own and the ctDNA-positive cases only partially overlapped the protein-biomarker-positive cases (Figs. 1 and 2A and Table 1).

Protein biomarkers have been combined in the past to achieve higher sensitivity (51). For example, nearly 20 y ago it was shown that combining CA19-9 and TIMP-1 was more sensitive for the detection of PDAC than either biomarker alone (34). More recently, it was shown that the combination of CA19-9, TIMP-1, and LRG-1 was more sensitive for the detection of early PDAC than CA19-9 alone (52). The combination of protein biomarkers with ultrasensitive ctDNA is by comparison novel. For example, a recent study evaluated a combination of ctDNA and CA19-9 for pancreatic cancer but found no benefit to combining the biomarkers over CA19-9 alone, perhaps due to inadequate sensitivity of the test used in detecting *KRAS* mutations (53). Furthermore, the specificity for ctDNA achieved in that study was relatively low, reducing its suitability for screening.

Our study demonstrates that the majority of patients with resectable pancreatic cancers can be detected through a noninvasive blood test. However, there are several limitations of the study that should be acknowledged. Patients with pancreaticobiliary disease, such as ascending cholangitis, cholecystitis, or chronic pancreatitis, can have high levels of CA19-9 (17). Our goal is to develop a test that will be used in healthy, asymptomatic individuals. Individuals

with ascending cholangitis, cholecystitis, or acute pancreatitis are almost always symptomatic. Incidentally detected chronic pancreatitis has been reported; however, the risk of this is extremely low, with an estimated prevalence of 0.49 per 100,000 (54–56), so this should not reduce specificity in practice. Another limitation is that, even though the majority of patients with resectable pancreatic cancer in our cohort could be detected, most patients still died from disease recurrence following surgery. These results make it clear that it will be necessary to detect cancers at earlier stages than those in our cohort to realize the maximum potential of earlier diagnosis, i.e., detection of individuals that can be cured by surgery alone.

On the other hand, the results we obtained may underestimate the survival benefits of early detection. The majority of the patients we studied, even though they had resectable cancers, were symptomatic, and their cancers were discovered only by virtue of their symptoms. Accordingly, 77% of patients in our cohort were stage IIB, and the median size of tumors in these patients was 3 cm. In a screening study of asymptomatic individuals, it is expected that a greater proportion of earlier-stage patients with smaller tumors will be discovered. This optimistic expectation is somewhat tempered by the fact that our combination assay was more sensitive for the detection of patients with larger tumors and patients with a poorer prognosis than for patients with smaller tumors, even though all tumors were surgically resectable (Fig. 1B, Table 2, Fig. S6, and Table S7). Another caveat to the use of this test for screening is that *KRAS* mutations are found in the circulation of patients with cancer types other than those of the pancreas, primarily cancers of the lung (57), and CA19-9, CEA, HGF, and OPN expression is elevated in several other cancer types (36, 58–60). Thus, patients testing positive would have to undergo appropriate imaging studies for tumor localization. Despite these limitations, our study establishes proof of principle for a large, prospective study in which these and other related issues can be evaluated.

The current study also lays a foundation for evaluation of patients at high risk for PDAC, which is a key strategy in implementation of early-detection technologies (61). As an example, new-onset diabetes is associated with an increased risk for pancreatic cancer. Approximately 1% of diabetic patients aged 50 y and older are diagnosed with pancreatic cancer within 3 y of first meeting criteria for diabetes (62). With an incidence of 1%, the positive predictive value/negative predictive value of the combination assay are expected to be 54% and 99.6%, respectively, in this population, which is well within the range of currently approved screening tests for cancers.

The applicability of our combination strategy to other cancers remains to be determined. Its success will largely depend on the extent of release of ctDNA from other cancer types coupled with the availability of protein and other biomarkers for those types. Available evidence indicates that many cancers have detectable ctDNA in their earliest stages, often more commonly than observed in pancreatic cancer (22). Similarly, a large number of protein biomarkers have already been described for the detection of numerous cancer types (16). In theory, these protein biomarkers could be thresholded in the way described here, offering cautious optimism for the use ctDNA–protein combinations to detect a variety of cancer types (22).

Materials and Methods

Detailed materials and methods are available in *SI Materials and Methods*. Briefly, DNA was purified from plasma using a QIAasymphony circulating DNA kit (catalog no. 1091063; Qiagen). Custom primers containing a unique identifier (UID) and amplicon-specific sequence (Table S9) were used to amplify plasma DNA, and the resulting products were sequenced on an Illumina MiSeq or HiSeq instrument. Protein biomarker plasma concentrations were determined using Luminex bead-based immunoassays on the Bio-Plex 200 platform (Bio-Rad). Plasma samples were scored as positive if the sample contained a *KRAS* mutation or if the concentration of CA19-9, CEA, HGF, or OPN was greater than 100 U/mL, 7.5 ng/mL, 0.92 ng/mL, or 158 ng/mL, respectively. All samples were

obtained following approval by the Institutional Review Boards for Human Research at The Johns Hopkins Medical Institutions, Walter and Eliza Hall Institute of Medical Research, Indiana University, Memorial Sloan-Kettering Cancer Center, University of Pittsburgh, Mayo Clinic, Asan Medical Center, University of Ulsan College of Medicine, San Raffaele Scientific Institute Research Hospital, and The University of Texas MD Anderson Cancer Center, and informed consent was obtained.

ACKNOWLEDGMENTS. We thank C. Blair and K. Judge from The Ludwig Center at Johns Hopkins for expert technical and administrative assistance and Dr. Dae Wook Hwang, Department of Hepatobiliary and Pancreas Surgery, Asan Medical Center, University of Ulsan College of Medicine;

J. Brooks, K. Chaffee, and W. Bamlet of the Mayo Clinic; and Dr. Herb Zeh of the University of Pittsburgh for their assistance with enrollment for this study. This work was supported by the Lustgarten Foundation for Pancreatic Cancer Research, The Virginia and D.K. Ludwig Fund for Cancer Research, The Conrad N. Hilton Foundation, The Sol Goldman Center for Pancreatic Cancer Research, The Michael Rolfe Pancreatic Cancer Research Foundation, The Joseph L. Rabinowitz Fund for Pancreatic Cancer Research, Susan Wojcicki and Dennis Troper, The Benjamin Baker Scholarship, The Commonwealth Foundation, National Health and Medical Research Council Grant APP1060804, The John Templeton Foundation, and NIH Grants P30-CA006973, P50-CA062924, P50-CA102701, CA06973, CA152753, CA196403, CA200468, GM-07309, and U01CA152753.

- Rahib L, et al. (2014) Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* 74: 2913–2921.
- Siegel RL, Miller KD, Jemal A (2016) Cancer statistics, 2016. *CA Cancer J Clin* 66:7–30.
- Ryan DP, Hong TS, Bardeesy N (2014) Pancreatic adenocarcinoma. *N Engl J Med* 371: 2140–2141.
- Ansari D, et al. (2017) Relationship between tumour size and outcome in pancreatic ductal adenocarcinoma. *Br J Surg* 104:600–607.
- Jung KW, et al. (2007) Clinicopathological aspects of 542 cases of pancreatic cancer: A special emphasis on small pancreatic cancer. *J Korean Med Sci* 22(Suppl):S79–S85.
- Egawa S, et al. (2004) Clinicopathological aspects of small pancreatic cancer. *Pancreas* 28:235–240.
- Ishikawa O, et al. (1999) Minute carcinoma of the pancreas measuring 1 cm or less in diameter—collective review of Japanese case reports. *Hepatogastroenterology* 46:8–15.
- Tsuchiya R, et al. (1986) Collective review of small carcinomas of the pancreas. *Ann Surg* 203:77–81.
- Howlader N, et al. (2016) SEER Cancer Statistics Review, 1975–2013 (National Cancer Institute, Bethesda, MD) Available at https://seer.cancer.gov/csr/1975_2013/. Accessed April 25, 2017.
- Bozic I, et al. (2013) Evolutionary dynamics of cancer in response to targeted combination therapy. *Elife* 2:e00747.
- Semrad TJ, Fahrni AR, Gong IY, Khatri VP (2015) Integrating chemotherapy into the management of oligometastatic colorectal cancer: Evidence-based approach using clinical trial findings. *Ann Surg Oncol* 22(Suppl 3):S855–S862.
- Moertel CG, et al. (1995) Fluorouracil plus levamisole as effective adjuvant therapy after resection of stage III colon carcinoma: A final report. *Ann Intern Med* 122:321–326.
- André T, et al. (2009) Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol* 27:3109–3116.
- Dy GK, et al. (2009) Long-term survivors of metastatic colorectal cancer treated with systemic chemotherapy alone: A North Central Cancer Treatment Group review of 3811 patients, N0144. *Clin Colorectal Cancer* 8:88–93.
- Huang AC, et al. (2017) T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. *Nature* 545:60–65.
- Liotta LA, Petricoin EF, 3rd (2003) The promise of proteomics. *Clin Adv Hematol Oncol* 1:460–462.
- Lennon AM, Goggins M (2010) Diagnostic and therapeutic response markers. *Pancreatic Cancer* (Springer, New York), pp 675–701.
- Clarke-Pearson DL (2009) Clinical practice. Screening for ovarian cancer. *N Engl J Med* 361:170–177.
- Locker GY, et al.; ASCO (2006) ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J Clin Oncol* 24:5313–5327.
- Haber DA, Velculescu VE (2014) Blood-based analyses of cancer: Circulating tumor cells and circulating tumor DNA. *Cancer Discov* 4:650–661.
- Dawson SJ, et al. (2013) Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med* 368:1199–1209.
- Bettegowda C, et al. (2014) Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 6:224ra24.
- Kinde I, et al. (2013) Evaluation of DNA from the Papanicolaou test to detect ovarian and endometrial cancers. *Sci Transl Med* 5:167ra4.
- Wang Y, et al. (2015) Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. *Sci Transl Med* 7:293ra104.
- Wang Y, et al. (2015) Detection of tumor-derived DNA in cerebrospinal fluid of patients with primary tumors of the brain and spinal cord. *Proc Natl Acad Sci USA* 112: 9704–9709.
- Wang Y, et al. (2016) Diagnostic potential of tumor DNA from ovarian cyst fluid. *Elife* 5:e15175.
- Springer S, et al. (2015) A combination of molecular markers and clinical features improve the classification of pancreatic cysts. *Gastroenterology* 149:1501–1510.
- Forshew T, et al. (2012) Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci Transl Med* 4:136ra68.
- Vogelstein B, Kinzler KW (1999) Digital PCR. *Proc Natl Acad Sci USA* 96:9236–9241.
- Dressman D, Yan H, Traverso G, Kinzler KW, Vogelstein B (2003) Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. *Proc Natl Acad Sci USA* 100:8817–8822.
- Vogelstein B, et al. (2013) Cancer genome landscapes. *Science* 339:1546–1558.
- Allen PJ, et al. (2017) Multi-institutional validation study of the American Joint Commission on Cancer (8th edition) changes for T and N staging in patients with pancreatic adenocarcinoma. *Ann Surg* 265:185–191.
- Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B (2011) Detection and quantification of rare mutations with massively parallel sequencing. *Proc Natl Acad Sci USA* 108:9530–9535.
- Zhou W, et al. (1998) Identifying markers for pancreatic cancer by gene expression analysis. *Cancer Epidemiol Biomarkers Prev* 7:109–112.
- O'Brien DP, et al. (2015) Serum CA19-9 is significantly upregulated up to 2 years before diagnosis with pancreatic cancer: Implications for early disease detection. *Clin Cancer Res* 21:622–631.
- Kim JE, et al. (2004) Clinical usefulness of carbohydrate antigen 19-9 as a screening test for pancreatic cancer in an asymptomatic population. *J Gastroenterol Hepatol* 19: 182–186.
- Uhlén M, et al. (2015) Proteomics. Tissue-based map of the human proteome. *Science* 347:1260419.
- Nazli O, Bozdogan AD, Tansug T, Kir R, Kaymak E (2000) The diagnostic importance of CEA and CA 19-9 for the early diagnosis of pancreatic carcinoma. *Hepatogastroenterology* 47:1750–1752.
- Di Renzo MF, Poulson R, Olivero M, Comoglio PM, Lemoine NR (1995) Expression of the Met/hepatocyte growth factor receptor in human pancreatic cancer. *Cancer Res* 55:1129–1138.
- Ikematsu S, et al. (2000) Serum midkine levels are increased in patients with various types of carcinomas. *Br J Cancer* 83:701–706.
- Koopmann J, et al. (2004) Evaluation of osteopontin as biomarker for pancreatic adenocarcinoma. *Cancer Epidemiol Biomarkers Prev* 13:487–491.
- Levina VV, et al. (2009) Biological significance of prolactin in gynecologic cancers. *Cancer Res* 69:5226–5233.
- Thorpe JD, et al. (2007) Effects of blood collection conditions on ovarian cancer serum markers. *PLoS One* 2:e1281.
- Jones S, et al. (2008) Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 321:1801–1806.
- Biankin AV, et al.; Australian Pancreatic Cancer Genome Initiative (2012) Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature* 491: 399–405.
- Waddell N, et al.; Australian Pancreatic Cancer Genome Initiative (2015) Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature* 518:495–501.
- Lennon AM, et al. (2014) The early detection of pancreatic cancer: What will it take to diagnose and treat curable pancreatic neoplasia? *Cancer Res* 74:3381–3389.
- Xie M, et al. (2014) Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med* 20:1472–1478.
- Jaiswal S, et al. (2014) Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med* 371:2488–2498.
- Genovesi G, et al. (2014) Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med* 371:2477–2487.
- Dong T, Liu CC, Petricoin EF, Tang LL (2014) Combining markers with and without the limit of detection. *Stat Med* 33:1307–1320.
- Capello M, et al. (2017) Sequential validation of blood-based protein biomarker candidates for early-stage pancreatic cancer. *J Natl Cancer Inst* 109:djx004.
- Le Calvez-Kelm F, et al. (2016) KRAS mutations in blood circulating cell-free DNA: A pancreatic cancer case-control. *Oncotarget* 7:78827–78840.
- Wilcox CM, et al. (2015) Chronic pancreatitis pain pattern and severity are independent of abdominal imaging findings. *Clin Gastroenterol Hepatol* 13:552–560, quiz e528–e559.
- Frøkjær JB, Olesen SS, Drewes AM (2013) Fibrosis, atrophy, and ductal pathology in chronic pancreatitis are associated with pancreatic function but independent of symptoms. *Pancreas* 42:1182–1187.
- Bahuva R, Walsh RM, Kapural L, Stevens T (2013) Morphologic abnormalities are poorly predictive of visceral pain in chronic pancreatitis. *Pancreas* 42:6–10.
- Herbst RS, Heymach JV, Lippman SM (2008) Lung cancer. *N Engl J Med* 359:1367–1380.
- Thomas DS, et al. (2015) Evaluation of serum CEA, CYFRA21-1 and CA125 for the early detection of colorectal cancer using longitudinal preclinical samples. *Br J Cancer* 113: 268–274.
- Di Renzo MF, et al. (1995) Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. *Clin Cancer Res* 1:147–154.
- Ei-Tanani MK, et al. (2006) The regulation and role of osteopontin in malignant transformation and cancer. *Cytokine Growth Factor Rev* 17:463–474.
- Kalinich M, et al. (2017) An RNA-based signature enables high specificity detection of circulating tumor cells in hepatocellular carcinoma. *Proc Natl Acad Sci USA* 114: 1123–1128.
- Chari ST, et al. (2005) Probability of pancreatic cancer following diabetes: A population-based study. *Gastroenterology* 129:504–511.