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Can circulating tumor and exosomal nucleic acids act as biomarkers for pancreatic ductal adenocarcinoma?

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Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with an extremely poor prognosis (5-year survival rate ~6%). Identifying biomarkers able to prognosticate and stratify patients will allow improved selection for operative resection or chemotherapy, and consequently better outcomes. PDAC is a heterogeneous disease characterized by an accumulation of molecular and genetic abnormalities. Activating mutations of the KRAS gene are mutated in 90% of PDAC cases and occur early in disease development. In this Editorial, we evaluate the study by Bernard *et al.* [1] which used blood samples as "liquid biopsies" from patients with localized and metastatic PDAC to isolate circulating tumor DNA (ctDNA) and exosomal DNA (exoDNA) in order to determine whether KRAS mutant allele fraction (MAF) in ctDNA and exoDNA was associated with survival outcomes. The authors revealed that exoDNA may be more useful than ctDNA alone, showing better concordance with tissue KRAS mutational status in treatment-naïve PDAC patients, predicting eventual surgical resectability, overall/progression-free survival and potentially anticipating tumor progression in patients with metastatic disease. The ability to monitor these as tumor markers could help monitor response to neoadjuvant chemotherapy in real time and identify disease progression during treatment cycles earlier than currently available clinical tests.

PDAC is a clinically silent disease with non-specific symptoms in its early stage. It is characterized by an accumulation of multiple genetic alterations in four common genes: *KRAS, TP53, SMAD4 and CDKN2A* [2]. Mutations in the KRAS gene are an early event in the development of PDAC [3], and detection of this gene either directly or via a surrogate marker at an early stage would be of great clinical significance.

In the last decade, several studies have measured circulating tumor DNA (ctDNA) in blood and other biofluids to detect cancer [4]. Exosomes are a specific subtype of extracellular vesicles of endocytic origin with a size range of 30-150 nm containing a cargo of nucleic acids, proteins and lipids. In cancer, they facilitate cell-to-cell communication [5] and the establishment of pre-metastatic niches [6]. In the evaluated study, Bernard *et al.* [1] used serial plasma samples to isolate ctDNA and exoDNA to determine their clinical utility as

biomarkers based on previous published work [7]. They also assessed whether their use in combination with serum CA19-9 might improve prognostication and therapeutic stratification of PDAC patients.

SUMMARY OF THE METHODS

In this study, Bernard *et al.* [1] collected plasma samples from 194 PDAC patients (April 2015 - October 2017). There were 2 cohorts consisting of 71 patients with localized disease and 123 with metastatic disease (confirmed either at surgery or through radiological investigation). A further 37 patients were included as controls; 25 diagnosed with pancreatic cysts and 12 with non-neoplastic pancreatic disease. All samples underwent isolation of cell-free circulating tumor DNA (ctDNA) and exosomal DNA (exoDNA) to assess the KRAS oncogene mutant allelic fraction (MAF) in both. Whole-blood samples were centrifuged at 2,500g for 10 minutes for plasma and an ultracentrifugation protocol was used to isolate exosomes. Both ctDNA and exoDNA was extracted using QIAamp Circulating Nucleic Acid mini kit and digital droplet PCR was used with a multiplex KRAS (codon 12 and 13) mutation assay. Baseline KRAS MAF was calculated and 34 patients from each cohort were available for longitudinal follow up whilst undergoing treatment (either surgery, chemotherapy consisting of gemcitabine and nab-paclitaxel (Abraxane) or FOLFIRINOX (folinic acid, 5-fluorouracil, irinotecan and oxaliplatin) or neoadjuvant chemoradiotherapy, using radiosensitizing gemcitabine/capecitabine at 30 or 50.4 Grays).

SUMMARY OF THE RESULTS

Detection of KRAS MAF was higher in exoDNA than in ctDNA for patients with PDAC. KRAS mutations were also detected in a small proportion of controls with pancreatic cysts (ExoDNA, 12%, n=3/25; ctDNA, 16%, n=4/25) and non-neoplastic pancreatic disease (ExoDNA, 25%, n=3/12; ctDNA, 17%, n=2/12). As expected, overall detection of KRAS MAF was found to be significantly higher in the metastatic cohort than those with localized disease, and was raised compared to patients with pancreatic cysts. Detection of KRAS was

compared with matched surgical tissue from 22 primary PDACs, and concordance was 95.5% and 68.2%, for exoDNA and ctDNA respectively. Concordance with 12 samples derived from fine needle aspirates was 83.3% and 66.8% for exoDNA and ctDNA, respectively.

Longitudinal assessment of exosomal KRAS MAF levels in localized PDAC patients correlates with surgical resectability

Serial liquid biopsies from 34 patients with localized disease taken before and after neoadjuvant chemotherapy appeared to demonstrate a correlation between changes in exoDNA KRAS MAF and surgical outcome. Patients who showed a reduction in exoDNA KRAS MAF from baseline went on to undergo surgery (70.6%; n=12/17), whilst a rise or no change was correlated with non-resectability (94.1%; n=16/17; *P*=0.0002). There was no significant correlation demonstrated with changes in ctDNA KRAS MAF. Additionally, the authors discussed a single index case where a rise in exoDNA KRAS MAF suggested progressive disease, but this was not identified until surgical exploration. This raises the interesting possibility that exoDNA may have a role to play in assessing patients with CT-occult PDAC progression. Of note, patients did not appear to have ^{18}F-FDG PET/CT scans to look for any metastatic disease. Analysis in conjunction with CA19-9 levels showed that in three patients, where no exoDNA KRAS was detectable, CA19-9 was able to predict clinical progression. Multivariate analysis in the localized cohort was not discussed.

High levels of KRAS MAF in liquid biopsies is associated with increased tumor burden and reduced survival in metastatic PDAC

Analysis of the metastatic cohort demonstrated no significant association between KRAS MAF in exoDNA or ctDNA with clinical characteristics. Within the metastatic cohort, the baseline measurement of ctDNA and exoDNA KRAS MAF was associated with significant reduction in PFS and OS. Furthermore, levels of both were also increased in patients with

liver metastases and larger metastatic burden. The authors also identified an association between poor performance status and greater KRAS MAF, but the cause for this is unclear.

ExoDNA and ctDNA in liquid biopsies predicts survival in treatment-naïve metastatic PDAC patients

A treatment-naïve subset of the cohort (n=104) was studied for the prognostic ability of liquid biopsy parameters at time of presentation. Using a Receiver-Operator Curve (ROC) analysis to determine a cutoff level, they determined this to be 5% MAF for exoDNA and 0% (presence/absence) of KRAS mutation for ctDNA. Kaplan-Meier analysis showed that these reaching these thresholds for ctDNA or exoDNA KRAS MAF were both associated with shorter PFS and OS. A CA19-9 level >300 was also associated with worse OS and trended towards reduced PFS. Multivariate analysis excluded ctDNA as an independent predictor of OS. Detectable ctDNA only became a significant determinant of OS when supported by either a CA19-9 level >300, or an exoDNA KRAS MAF >5%.

Plasma peaks in exoDNA KRAS MAF precedes disease progression in metastatic PDAC

Serial blood samples from 34 patients with metastatic disease (mixture of treatment naïve and on-treatment patients) were followed up for a median of 202 days. Of these, 59% (n=20/34) progressed on therapy with a median time to progression of 176 days. Patients that did not progress were followed up for a median of 300 days. ROC analysis revealed that a peak exoDNA KRAS MAF >1% in any “on-treatment” blood draw was significantly associated with disease progression. Analysis of the ctDNA levels was unable to determine this. A rise of 20% in CA19-9 levels gave a sensitivity and specificity of 70% and 89% in predicting progression of disease respectively. ExoDNA KRAS MAF >1% had a greater sensitivity and specificity of 79% and 100% respectively. Furthermore, the exoDNA KRAS MAF appeared to peak at a greater lead time (i.e. prior to radiological progression) than CA19-9 levels.

COMMENTARY

Bernard *et al.* [1] performed a large prospective study of patients with PDAC that has shown the clinical usefulness of exoDNA in plasma as a marker to prognosticate patient outcomes. The measured fraction of mutant allele KRAS in exoDNA alone proved to be a good predictor of response to neoadjuvant chemoradiotherapy and surgical resectability in patients with localized PDAC. In metastatic disease, exoDNA was associated with shorter PFS and OS, and was more reliable than ctDNA. This study also demonstrated these markers ability to longitudinally monitor patients. Changes such as detectable ctDNA and exoDNA KRAS MAF levels were correlated with patient outcomes with an improved lead time of 50 days over current markers, such as serum CA19-9. When the average life expectancy of patients with advanced PDAC is 6 months, this would allow earlier therapeutic intervention and reduce chemotherapy-related morbidity [8].

The relative failure of ctDNA to effectively track response to chemotherapy may be due to the “stochastic nature of circulating nucleic acids” (i.e. intra-patient heterogeneity) and chemotherapy has been shown to create a confounding increase in ctDNA, not mirrored in exosomes [9]. Interestingly, in this study population there were several false positives noted within the control group. Detectable ctDNA KRAS MAF has been previously noted in studies at a rate of between 3.7-14.8% [7,10,11], which reiterates the difficulties of biomarker specificity.

Sensitivity of this study was limited by using a multiplex KRAS array, rather than a broader tumor gene panel, which excluded patients with wild-type KRAS or hotspot mutations in codon 61. Their overall detection rate of KRAS mutation in blood plasma was relatively low compared to the literature and this could lead to a bias in the overall concordance. Whether this was due to their choice of assay (covering only 80% of known PDAC mutations) or sample bias is uncertain. However, the concordance of 95.5% for exoDNA KRAS with tissue KRAS mutation status in treatment-naïve PDAC patients remains an impressive result,

highlighting the potential of exosomal nucleic acid measurement to give us accurate tumor-specific information.

The paper by Bernard *et al.* [1] has shown that potential nucleic acid markers within exosomal cargo may be able to complement currently validated tools, such as serum CA19-9, as well as providing added diagnostic and/or prognostic value. Circulating cell-free tumor DNA may have a limited use as it is susceptible to relatively rapid plasma nuclease degradation and/or elimination through various pathways (e.g. liver or kidney) [12] and there is some evidence that the greater proportion of ctDNA in plasma is actually exosomal [13]. Exosomes in comparison are known to be stable through freeze-thaw cycles with minimal loss of cargo, making them suitable for further clinical biomarker research [14]. Circulating cell-free tumor DNA has been shown to be heavily fragmented and unequally representative of the genome, which is likely to have accounted for some of the mismatch between tissue-detectable mutations and ctDNA [15].

As more is discovered about exosomes in cancer, there has been a great interest in smaller cargo such as microRNAs (miRNA) and other RNAs in blood and biofluids as biomarkers (**Table 1**). Most recent studies have focused on exosomal miRNAs (~22 base pairs), but exosomal long coding and non-coding RNAs (>200 base pairs) have also been found. Exosomal miRNAs have been shown to play a role both in PDAC tumor microenvironment interactions (e.g. inducing cell proliferation; promoting angiogenesis; promoting matrix remodeling via protease secretion [16], and in metastatic spread and growth [17]). Indeed, characterizing these signaling markers early during tumor proliferation might enable this deadly disease to be detected sooner and stratified better.

The development of other biofluid-based biomarkers in PDAC has also turned to bile as a source of exosomes which should enable greater organ-specificity given their proximity to the malignant lesion [18] and may avoid the difficulty of differentiating plasma exosomes (i.e. ensuring the exosomes isolated are from the organ / cancer of interest) [19]. It is likely that with further developments in understanding the PDAC “secretome”, clinicians will be able to

use a complement of exosomal RNA/DNA assays as a non-invasive liquid biopsy to assist in clinical decision-making.

KEY ISSUES:

- Plasma exosomal and circulating KRAS mutant allele fraction (MAF) can be used as potential biomarkers which correlate with tumor progression and outcomes in patients with PDAC.
- ExoDNA KRAS MAF shows better concordance with tissue KRAS mutational status in treatment-naïve PDAC patients compared to ctDNA KRAS MAF.
- Serial measurement of exoDNA KRAS MAF levels in localized PDAC patients correlates with eventual surgical resectability after neoadjuvant chemotherapy.
- A threshold of 5% exoDNA KRAS MAF or the detection of a ctDNA KRAS mutation were both associated with shorter PFS and OS in PDAC patients with metastatic disease.
- In metastatic patients, an increase in exoDNA KRAS MAF >1% during treatment was significantly associated with further disease progression.
- ExoDNA KRAS MAF was an earlier marker of tumor progression than serum CA19-9 levels.
- Future directions for research should include the examination of exosomal RNA and DNA cargo in blood and other biofluids from PDAC patients in order to develop better biomarkers.

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Declaration of interest

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Table 1: Previous studies investigating biofluid exosomal nucleic acids in pancreaticobiliary cancers

Exosomal DNA studies

First Author & Ref.	Year	No. of Patients	Biofluid	Biomarker	Significant comments
Yang et al [20]	2017	PDAC (n=48), IPMN (n=7), CP (n=9), Controls (n=114)	Serum	**$KRAS^{G12D}$ and $TP53^{R273H}$**	In PDAC patients, digital PCR analyses of exosomal DNA identified $KRAS^{G12D}$ mutation in 39.6% of cases, and $TP53^{R273H}$ mutation in 4.2% of cases.
Allenson et al [7]	2017	Discovery: PDAC all stages (n=68), PDAC localized (n=20), Controls (n=54) Validation: PDAC early stage (n=39), Controls (n=82)	Plasma	**Mutant KRAS allelic fraction (MAF)**	In the validation cohort, mutant KRAS exoDNA was detected in 43.6% of early-stage PDAC patients and 20% of healthy controls. Higher KRAS MAF was also associated with reduced Disease Free Survival (DFS) in patients.
Kahlert et al [21]	2014	PDAC (n=2), Controls (n=2)	Serum	**Mutations in KRAS and p53**	Study provides evidence that exosomes can carry large fragments (~10 kb) of double-stranded genomic DNA.

Exosomal long non-coding RNA studies

First Author & Ref.	Year	No. of Patients	Biofluid	Biomarker	Significant comments
Li et al [22]	2018	PDAC (n=20) Control (n=20)	Plasma	**lncRNA-Sox2ot upregulated**	Expression of Sox2ot was significantly associated with TNM stage (*P*= 0.014) and was also related to lymphatic and vascular invasion. Sox2ot competitively binds to the miR-200 family to regulate the expression of Sox2, thus promoting invasion and metastasis of PDAC
Ge et al [23]	2017	CCA (n=35), Controls (n=56)	Bile	**ENST00000588480.1 and ENST00000517758.1 upregulated**	Combined Sensitivity was 82.9% (AUC: 0.709; 95% CI, 0.6010.817). Increasing levels tended to be associated with the advancing TNM stage.

Exosomal coding RNA studies

First Author & Ref.	Year	No. of Patients	Biofluid	Biomarker	Significant comments
Kitagawa et al [24]	2018	PDAC (n=27) Controls (n=13)	Serum	**2 mRNAs (WASF2, ARF6) and 2 snoRNAs (SNORA74A, SNORA25)**	The AUCs of WASF2, ARF6, SNORA74A, and SNORA25 in serum from patients in the early stages of PDAC (stages 0, I, and IIA) were > 0.90, compared with an AUC of 0.93 for serum CA19-9
Exosomal microRNA studies					
First Author & Ref.	**Year**	**No. of Patients**	**Biofluid**	**Biomarker**	**Significant comments**
Zhou et al [25]	2018	Training: PDAC/Control (n=40, n=40) Testing: PDAC/Control (n=112, n=116) Exosomal: PDAC/Control (n=31, n=37)	Plasma	**miR-122-5p and miR-193b-3p were up-regulated, while miR-221-3p was down-regulated**	The AUCs for exosomal miR-122-5p (0.722; 95% CI: 0.591–0.853), exosomal miR-193b-3p (0.651; 95% CI: 0.51–0.792) and the signature of the two exosomal miRNAs combined (0.849; 95% CI: 0.756–0.942).
Wang et al [17]	2018	PANC-1 and BxPC-3 PDAC cells	Serum	**miR-301a3p**	Shown to predict TNM classification.
Takahasi et al [26]	2018	PDAC (n=56) Control (n=3)	Plasma	**miR-451a**	Divided into high and low expression. Positively associated with tumor size, stage, negatively associated with disease free (P=0.004) and overall survival (P=0.001).
Li et al [27]	2018	PDAC (n=93)	Plasma	**Circ-PDE8A**	Up-regulation was significantly associated with lymphatic invasion (*P*=0.014), T factor (*P*=0.049) and TNM stage (*P* =0.005).
Li et al [28]	2018	PDAC (n=73)	Plasma	**miR-222**	High in PDAC patients and significantly correlated to tumor size and TNM stage, and was an independent risk factor for survival.
Li et al [29]	2018	PDAC (n=40)	Plasma	**Circ-IARS**	Circ-IARS associated with tumor vessel invasion, liver metastasis, and TNM stage. It was also shown to competitively bind miR-122, inhibit its expression and release inhibition of downstream target gene RhoA activity, increase the expression of F-actin, and promote cell contraction.
Goto et al [30]	2018	PDAC (n=32), IPMN (n=29), controls (n=22)	Serum	**miR-191, miR-21 and miR-451a**	Significantly up-regulated in patients with PDAC and IPMN compared to controls (*P*<0.05). The AUC, diagnostic accuracy and specificity of the 3 exosomal miRs were superior to circulating miRs. However, CA19–9 was still superior for the diagnosis of advanced PDAC.
Bartsch et al [31]	2018	PDAC (n=96) Controls (n=20)	Serum	**The panel miR-196b/LCN2/TIMP1**	Could distinguish high-grade lesions and stage I PDAC from controls with absolute specificity and sensitivity.

Xu et al [32]	2017	PDAC (n=15) Controls (n=15)	Plasma	**miR-196a and miR-1246**	These were enriched in localized PDAC. Immunoaffinity isolation using GPC-1 antibodies for plasma exosome miRNA analysis did not improve results.
Mikamori et al [33]	2017	PDAC (n=23)	Plasma	**miR-155**	Up-regulation correlated with reduced DFS, but not OS and could be used as a clinical marker in gemcitabine resistance.
Lai et al [34]	2017	PDAC (n=29) CP (n=11) Controls (n=6)	Plasma	**miR-10b, -20a, -21, -30c, -106b, and -181a significantly higher** **miR-let7a and miR-122 were lower**	
Chen et al [35]	2017	PC (n=16) CP (n=18) Controls (n=20)	Serum	**miR-23b-3p**	Was verified to be the only up-regulated miRNA in both PDAC and CP groups, as compared to normal controls.
Machida et al [36]	2016	PDAC (n=6), Controls (n=6)	Saliva	**miR-1246 and miR-4644**	The AUCs of both were >0.70, indicating fair discriminatory power.
Madhavan et al [37]	2015	PDAC (n=131) CP (n=25) Benign disease (n=34) Control (n=30)	Serum	**miR-1246, miR-4644, miR-3976, and miR-4306**	
Li et al [19]	2014	CCA (n=46), Controls (n=50)	Bile	**A panel of 5 microRNAs: miR-191, miR-486-3p, miR-1274b, miR-16, miR-484**	Sensitivity 67% and specificity 96%.
Que et al [38]	2013	PDAC (n=22) Benign (n=6) Ampullary (n=7) CP (n=6) Controls (n=8)	Serum	**miR-17-5p and miR-21**	AUC of miR-17-5p and miR-21 were 0.887 (0.796 to 0.978) and 0.897 (0.803 to 0.991), respectively.
Shigehara	2011	PDAC (n=9),	Bile	**miR-9, miR-145*,**	Setting the specificity threshold to 100% showed the sensitivity level to be 88.9% for miR-9, miR-302c*, miR-

et al [39]		Controls (n=9)		**miR-105, miR-147b, let-7f-2*, let-7i*, miR-302c*, miR-199a-3p, miR-222* and miR-942**	199a-3p, and miR-222*; 77.8% in miR-145*, miR-105, and miR-942; and 66.7% in miR-147b, let-7f-2*, and let-7i.
KEY: CCA, cholangiocarcinoma; PDAC, pancreatic ductal adenocarcinoma; IPMN, Intraductal Papillary Mucinous Neoplasm; CP, chronic pancreatitis					