Commentary

Mutations in KRAS: are they a valid biomarker for pancreatic ductal adenocarcinomas diagnosis?

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Introduction

Of all cancers, pancreatic cancer is associated with the most detrimental clinical outcome. Even when treated according to most recent guidelines, more as ninety percent of patients will not survive the cancer beyond one to five years after diagnosis (1). Compounding the clinical problem posed by pancreatic cancer is its sheer size: no less as 337,872 cases were reported in 2012 and the number of cases is still increasing every year (2). A major factor in combating pancreatic cancer is the difficulty in identifying and differentiating the types of pancreatic cancers at an early stage. The present identification for this cancer is through computed tomography (CT), endoscopic retrograde cholangiopancreatogram (ERCP), Endoscopy ultrasound (EUS) followed with EUS-Fine needle aspiration biopsy (FNAB) and pathology (3), but optimism in this regard is growing due to the revolution in biomarker field per se. Molecular markers currently under assessment for their usefulness for the early diagnosis of pancreatic cancer include those based on the detection of genetic abnormalities within the mutational spectrum of pancreatic cancer, the detection of activated forms of signaling kinases involved in the progression of pancreatic cancer, the presence of specific miRNA variants in specific bodily fluids, distinct epigenetic alterations, as well as the finding of DNA with aberrant telomere length in fluids in contact with potential pancreatic cancers (4-6). The four major genes involved in the mutational spectrum for pancreatic ductal adenocarcinoma (PDAC) include P16, KRAS, TP53, and SMAD4 (1,7). Molecular markers involving detection of genetically aberrant variants of these genes or otherwise may aid making a definitive diagnosis of PDAC but may also guide gauging the potential response to chemotherapy and/or radiation therapy, thus improving success rates and help avoiding subjecting patients to side effect-prone treatment modalities (8). They may also guide assessing the extent of tumor heterogeneity during the course of cancer and thus the potential chances for success of treatment with targeted therapy like biological or novel kinase inhibitors (8). Sampling of material for determining biomarkers in suspected or established PDAC can be challenging: liquid biopsies (blood etc.), feces or saliva contain only minor amounts of tumor material, EUS-FNAB is invasive and produces also relatively little material and PDAC surgery is also challenging and the long time the procedure takes compromises quality of the biological material obtained. This situation is alleviated somewhat by the recent advent of technology capable of nanoscale DNA isolation and its analyses by modified new generation sequencing (NGS), digital PCR and QPCR while conversely better endoscopic procedures improve the quantity of material biopsy collected as well as the safety of the procedure

involved. In conjunction these developments are starting to revolutionize the field but proof-of-principle studies are still relatively scarce, especially with regard to PDAC but also with cancerous disease in general (9-14). Important directions in the field aimed at providing such proof-of-principle for PDAC include nanoscale measurement of circulating or pancreatic cyst fluid cell free DNA (cfDNA), detection of circulating tumor cells (CTC) and analysis of exosomes from the blood. KRAS is ubiquitously mutated in PDAC and constitutes thus a rational target gene in this respect. *Table 1* explains in detail of the different non-invasive methods that are under study and critical analysis involving KRAS and other genes which can be used as diagnostic and prognostic markers for PDAC.

KRAS mutational spectrum in diagnosis of pancreatic cancer

An important advance in respect is that described by Le Calvez-Kelm *et al.* in a recent issue of oncotarget (15), who report on the use of KRAS mutation detection using as low as 2 ng of cfDNA with deep sequencing and Needlestack variant caller algorithm analysis from the patients with PDAC, chronic pancreatitis, and healthy controls. The study constitutes the largest screening of KRAS mutations in plasma samples of pancreatic cancer cases hitherto and its comparison to, other pathological pancreatic conditions and healthy controls allows for the comprehensive assessment of sensitivity and specificity of KRAS mutations using cfDNA. In this study the authors tried to understand how the KRAS mutations at the codon's 12, 13 and 61 and new non-hotspot codons can affect the outcome of the disease as well to use it as the prospective diagnostic biomarker along in comparison to conventional plasma CA19.9 levels. Technically the study was exemplary as authors were able to identify with 0.08% of allele fraction detectability KRAS mutation status. Importantly, however, although the overall sensitivity in combination with CA19.9 increases to 95%, a limited sensitivity of 21.1% for the detection of pancreatic cancer was observed, substantially lower in comparison to CA19.9 alone. This would suggest that measuring cfDNA is not a promising avenue here and thus goes against the general direction of the field (see *Table 1*). The authors convincingly argue that other studies showing high specificity with KRAS did not include healthy controls (15). This method of specific amplicons obtained from a 2 ng DNA at the regions of exon2 and exon3 for the Kras hot spot codons are from exon 2 and exon 3 i.e. codon 12, codon 13 and codon 61, and non-hotspot codons 59, 62, 64 and 70 is an robust method to be used for other somatic mutations as in EGFR, TP53, SMAD4 and other DNA markers with in pancreatic cancer. Hence amplicon-based KRAS mutations sequencing is not as specific as anticipated, an important observation forcing the field to explore radically different avenues in this respect, e.g., microbiome determinations or mass spectrometry based directions.

Advantages and drawbacks of using KRAS and other mutational spectrum for diagnosis

Bailey *et al.*, in the recent study have shown that the classification of different types of pancreatic cancer can be asserted using KRAS mutation and upregulation. The aberrantly differentiated endocrine exocrine origin PDAC's carry the upregulated KRAS than other squamous, pancreatic progenitor and immunogenic tumors of pancreas (16). Kadayifci *et al.* showed the difference between the pancreatic cysts can be studied using DNA isolated from Cyst Fluids. They were able to obtain an overall accuracy of 86.2% when GNAS, KRAS and carcinoembryonic antigens determinations were combined, but this accuracy was markedly superior to when the individual determinations were used stand alone (17). In another study by Deshpande *et al.*, the authors were able to identify a difference between pancreatic intra/epithelial tumors and the tumors driven into pancreatic duct from common bile duct origin using KRAS mutational analysis (95% *vs.* 11%) (18), thus although mutational analysis of cfDNA was very disappointing in the study Le Calvez-Kelm *et al.*, analysis of mutation state per se will remain useful for diagnostic purposes when cellular material can be obtained.

Pancreatic cancers are among the most versatile forms of oncological disease. Preclinical work suggests that epithelial mesenchymal transition and the subsequent dissemination of tumor cells in the body is a very early event in this cancer, which already takes before obvious tumor formation and clear micrometastatic invasion into nearby tissues and lymph nodes can be detected (19). Encouragingly, work in colon cancer suggests that epithelial mesenchymal transition may be sensitive to various pharmacological inhibitors [e.g., ROCK inhibitors (20)] and thus very early detection through screening in combination with anti epithelial

Table 1 Non-invasive biomarkers studies in pancreatic and other cancers involving cfDNA, CTCDNA, and exosomes

Cancer	DNA	Methodology	Inference		References
			Sensitivity	Specificity	
Pancreatic cancer	Circulating tumor cells	NGS	NA	NA	Premasekharan G *et al.*; Cancer Lett 2016;380:144-52. DOI: 10.1016/j.canlet.2016.06.017
		Digital PCR	36% for G12D, 50% for G12V, and 0/5 for G12C in plasma samples VS 72% (44% G12D, 20% G12V, and 10% G12C) for the positive tumor DNA samples	NA	Brychta N *et al.*; Clin Chem 2016;62:1482-91. DOI: 10.1373/clinchem.2016.257469
		Immunomagnetic separation	47.1% CTC in all pancreatic cancers	100% CTC in all pancreatic cancers	de Albuquerque A *et al.*; Oncology 2012;82:3-10. DOI: 10.1159/000335479
		Digital PCR	20% of CTC *vs.* 26% with KRAS on cf DNA	NA	Earl J *et al.*; BMC Cancer 2015;15:797. DOI: 10.1186/s12885-015-1779-7
	Circulating Cell free DNA	NGS	KRAS - 10.3 % (local), 17.5% (regional) 33.3% (systemic)	KRAS 98.2% (codon 12,13,61) and 96.4% (any other site)	Le Calvez-Kelm F *et al.*; Oncotarget 2016. DOI: 10.18632/oncotarget.12386
		NGS	KRAS - 25% (cf DNA *vs.* tumor tissue DNA)	NA	Pishvaian MJ *et al.*; Oncotarget 2016. DOI: 10.18632/oncotarget.13225
		NGS	43% cfDNA of localized cancer	NA	Sausen *et al.*; Nat Commun 2015;6:7686. DOI: 10.1038/ncomms8686
		NGS	KRAS, TP53, APC, FBXW7, and SMAD4 92.3% average sensitivity	KRAS, TP53, APC, FBXW7, and SMAD4 100%	Zill OA *et al.*; Cancer Discov 2015;5:1040-8. DOI: 10.1158/2159-8290.cd-15-0274
		Digital PCR	31% (cf DNA *vs.* tumor tissue DNA)	NA	Hadano N *et al.*; Br J Cancer 2016;115:59-65. DOI: 10.1038/bjc.2016.175
		Digital PCR	KRAS 62.6% cfDNA	NA	Kinugasa *et al.*; Cancer 2015;121:2271-80. DOI: 10.1002/cncr.29364
		Digital PCR	KRAS 29.2% cfDNA	NA	Takai *et al.*; Sci Rep 2015;5:18425. DOI: 10.1038/srep18425
		Quantitative PCR	NA (cf DNA ALU repeats)	NA (cf DNA ALU repeats)	Sikora K *et al.*; Int J Biol Markers 2015;30:e136-41. DOI: 10.5301/jbm.5000088.
	Cell free DNA in Cyst fluids	Quantitative PCR	NA (cf DNA ALU repeats)	NA (cf DNA ALU repeats)	Utomo WK *et al.*; Am J Cancer Res 2016;6:1837-41
	Exosomes miRNA	Quantitative PCR and Microarray	81% (miR-1246, miR-3976, miR-4306, miR-4644)	94% (miR-1246, miR-3976, miR-4306, miR-4644)	Madhavan B *et al.*; Int J Cancer 2015;136:2616-27. DOI: 10.1002/ijc.29324

Table 1 *(continued)*

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Cancer	DNA	Methodology	Inference		References
			Sensitivity	Specificity	
Other cancers-colorectal, melanoma and NSCLC-	Circulating Cell free DNA	droplet digital PCR	KRAS - 84% (cf DNA compared to FFPE DNA); 95% when cf DNA concentration increased	88%(cf DNA compared to FFPE DNA)	Janku F *et al.*; Ann Oncol 2016. DOI: 10.1093/annonc/mdw670

Studies which may aid understanding the driver and passenger mutations in PDAC through cfDNA of the patients, using modified NGS techniques which have high sensitivity to identify the low frequency alleles, together with digital PCR and QPCR approaches. These methods are used for measuring biomarkers of DNA from CTC, CF, and exosomes. This table lists published reports on non-invasive approaches.

mesenchymal transition in high risk individuals remains an attractive proposition, despite the apparent difficulties in performing such screening through KRAS mutational analysis of cfDNA.

Nanoscale measurements of cfDNA and other DNA/RNA/proteins through non-invasive methods

Huang *et al.*, showed that the use of AMP-based NGS methodology allows the detection of variants in allele frequencies as low as 1% and compared with allele-specific PCR as well digital PCR (1–5%) for KRAS, TP53, and SMAD4 in pancreatic cancer patients (21). There are other studies which may aid understanding the driver and passenger mutations in PDAC through cfDNA of the patients, using modified NGS techniques which have high sensitivity to identify the low frequency alleles, together with digital PCR and QPCR approaches. These methods are used for measuring biomarkers of DNA and RNA from CTC, CF, and exosomes (*Tables 1*,*2*). *Table 2* gives the insights of the non-invasive non-DNA marker studies, which are aimed at improving pancreatic cancer diagnosis and prognosis. The same methods are being used in other cancers too like, colorectal, lung, liver and breast cancers in which such nanoscale measurements are showing some promise towards establishing cancer prognosis and may become useful for better therapy design. The study by Le Calvez-Kelm *et al.* shows that achieving this in PDAC may still entail a prolonged effort.

Conclusions

Pancreatic cancer with its associated infaust prognosis urgently needs better early detection of disease, especially for screening of high risk individuals. Despite early promise and theoretical considerations, however, KRAS mutional analysis of cfDNA seems not a way forward here. A more multifaceted panel of mutational spectrum biomarkers study (KRAS, TP53, SMAD4, P16, EGFR GNAS, MENN1, DAXX, VHL and in combination of pancreatic cancer specific STR markers) along with epigenetic alterations in cfDNA in pancreatic cancer patients may still provide a successful prognostic and diagnostic avenue, but generally speaking cfDNA does not appear very promising direction. Hence focus should now be directed to alternative methodology.

Table 2 Non-invasive non-DNA biomarkers studies in pancreatic cancer

Cancer	Other Biomolecules	Methodology	Inference		References
			Sensitivity	Specificity	
Pancreatic cancer	miRNA	Quantitative PCR and Microarray	81% (miR-1246, miR-3976, miR-4306, miR-4644)	94% (miR-1246, miR-3976, miR-4306, miR-4644)	Madhavan B *et al.*; Int J Cancer 2015;136:2616-27. DOI: 10.1002/ijc.29324
		microRNA array-based	59.3% (miR-744/cel-miR-39)	89.6% (miR-744/cel-miR-39)	Miyamae M *et al.*; Br J Cancer 2015;113:1467-76. DOI: 10.1038/bjc.2015.366
	Long non coding RNA	Arraystar Human LncRNA Microarray	89% (HOTTIP-005 to HDRF), 75.6% (RP11-567G11.1 to RDRF)*	68.3% (HOTTIP-005 to HDRF), 66.7% (RP11-567G11.1 to RDRF)*	Wang Y *et al.*; Oncotarget 2015;6:35684-98. DOI: 10.18632/oncotarget.5533
	Epigenetics	Other techniques	81.7% (Normal *vs.* Chornic pancreatitis) 91.2% (pancreatic cancer *vs.* Chornic pancreatitis)	78% (Normal *vs.* Chornic pancreatitis) 90.8% (pancreatic cancer *vs.* Chornic pancreatitis)	Liggett T *et al.* Cancer 2010;116:1674-80. doi: 10.1002/cncr.24893
	Proteins	Transmission electron microscopy (immunogold labelled GPC1) and flow cytometry (FITC labelled GPC1)	100%	100%	Melo SA *et al.*; Nature 2015;523:177-82. doi: 10.1038/nature14581

Studies which may aid understanding the driver and passenger mutations in PDAC through cfDNA of the patients, using modified NGS techniques which have high sensitivity to identify the low frequency alleles, together with digital PCR and QPCR approaches. These methods are used for measuring biomarkers RNA and protein from CTC, CF, and exosomes. This table lists published reports on these non-DNA biomarkers. *, HDRF and RDRF are the lncRNA fragments of HOTTIP-005 and RP11-567G11.1 respectively present in serum.

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