Published in final edited form as:

Pancreat Disord Ther. 2015 June ; 5(2): . doi:10.4172/2165-7092.1000156.

Circulating DNA and Micro-RNA in Patients with Pancreatic Cancer

Eveline E Vietsch¹, Casper HJ van Eijck², and Anton Wellstein^{1,*}

¹Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington DC, USA ²Department of Surgery, Erasmus Medical Center, Rotterdam, The Netherlands

Abstract

Collecting repeat samples of blood (“liquid biopsies”) is a broadly used clinical approach for serial monitoring of disease or response to treatments. In patients with cancer the most distinct molecular feature are somatic mutations acquired by cancer cells present in the diseased tissue. Indeed, mutant DNA derived from dying or lysed cancer cells can be isolated from patient serum samples, subjected to DNA sequencing and to analysis of abundance as a measure of tumor burden. Also, changes in the DNA mutation patterns in serum samples collected over time can indicate altered pathways or clonal evolution of the disease and altered abundance of mutant DNA suggests an altered disease burden. In addition, during the course of treatment, changes in circulating DNA mutation patterns can indicate the emergence of resistant clones and prompt changes in treatment. In contrast to mutant DNA, microRNAs (miR) are transcribed, processed, packaged and released from cells in normal and in diseased tissues as part of the extracellular crosstalk between cells. Interestingly, released miR can function in cell-to-cell communication and as hormone-like signals that operate at a distance through their release into the circulation and subsequent uptake into cells in distant tissues. Circulating miR expression patterns can be established from serial serum samples and monitored for alterations over time. Circulating miR provide a readout of the organism’s steady state and serial analyses will indicate changes in the response to therapy or an altered physiologic or disease state. Furthermore, changes in circulating miR patterns can indicate treatment efficacy or resistance as well as adverse effects associated with the respective intervention. Thus, the combined serial analysis of mutant DNA and miR in the circulation has the potential to provide a molecular footprint of pancreatic cancer and can be used to monitor treatment responses or resistance to treatment in real time with a minimally invasive procedure.

Keywords

Pancreatic cancer; Liquid biopsy; Biomarkers; Circulating DNA; Circulating microRNA; Prognostic markers; Treatment response

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

*Corresponding author: Anton Wellstein, Georgetown University Medical School, Lombardi, Comprehensive Cancer Center, Research Building; Room E311, 3970 Reservoir Road, Washington DC, Tel: +1202687-3672, anton.wellstein@georgetown.edu.

Introduction

Pancreatic cancer is a deadly malignant disease with surgical resection currently the only potentially curative modality although only 10–20% of patients are diagnosed with a resectable tumor [1,2]. Patients with advanced or metastatic disease receive chemotherapy with either gemcitabine or S-1, an oral fluoropyrimidine, or with the combination treatment FOLFIRINOX [3–5]. The prognosis of patients receiving surgery and chemotherapy is dependent on a number of characteristics including radiographical staging, histological grading, ECOG scores and CA19-9 levels [6–8]. However, very little improvement in the outcomes of patients with pancreatic cancer has been seen in the past 30 years [1]. Here we will review the potential to use blood samples or “liquid biopsies”, a recent term coined to indicate the relation to cancer tissue analysis, as markers of genetic characterization of a cancerous lesion. It is conceivable that blood samples can reflect the mutation profile of a primary cancer as well as residual cancer cells that are not accessible physically or below detection. Additionally, liquid biopsies can be taken repeatedly to follow evolution of the disease. Identification of specific pancreatic cancer markers is crucial for the improvement of controlling this disease and recent studies have shown that circulating mutant DNA can be found in the majority of cancer patients and can provide a measurement of tumor burden. Moreover, it could be used to identify specific mutant genotypes emerging from a heterogeneous tumor population. In contrast to the unique source of mutant DNA, i.e. cancer cells, miR isolated from the circulation is derived from virtually all tissues in the organism (Figure 1.) The analysis of expression patterns of circulating miR will thus provide a footprint of the steady state of the organism that encompasses normal as well as pathologic processes. Also, it will reflect the impact of therapeutic interventions on the diseased as well as healthy tissues. Here we review the published data on circulating nucleic acids, i.e. DNA and miR that reflect the disease state and treatment responses of patients with pancreatic cancer.

Circulating cell-free DNA (cfDNA)

The occurrence of cell-free, circulating DNA (cfDNA) in the blood of patients was first described several decades ago [9–12]. Whilst the concentration of cfDNA in the bloodstream of healthy individuals is very low, cfDNA abundance increases significantly due to pathological processes, as reviewed by Anker et al. [13]. Elevated levels of cfDNA are detected in patients after surgery and trauma [14], myocardial infarction [15–17], rheumatoid arthritis [9], pancreatitis [18] and intensive care related conditions [19,20]. Additionally, athletes have increased levels of cfDNA after heavy exercise [21] and pregnant women carry fetal DNA in their bloodstream that has been used in prenatal diagnostics to detect fetal abnormalities [22–24].

Despite the multitude of studies describing cfDNA, the origin and nature of the double-stranded nucleic acid fragments isolated from the circulation remain ambiguous [25,26]. It is commonly accepted that apoptotic cells release DNA into the bloodstream. During apoptosis DNA is cleaved and packaged into nucleosomes of roughly 180 base pairs [27,28] and analysis of the size of circulating DNA indicates that at least some is from debris after the

apoptotic process [29–32]. Additionally, there is some evidence of lymphocytes and cancer cells shedding DNA by unknown mechanisms [25,32–35]. Even though the complex origin of cfDNA needs to be further elucidated, elevated levels of cfDNA have been shown in independent studies of cancer patients when compared to healthy individuals. This elevated cfDNA is indeed mostly derived from cancer cells [10,11,30,36–39].

Factors impacting circulating mutant DNA levels

It is widely accepted that circulating mutant DNA originates from cancer cells. However as studies accumulate, it becomes clear that a multitude of aspects contribute to the varying levels of mutant DNA. Firstly, biological processes influence the overall quantity of circulating DNA. Circulating mutant KRAS DNA can be excreted by the kidneys and can be detected in the urine [40]. Plasma nucleases [41] and DNase1 [42] can contribute to the degradation of circulating DNA. Furthermore, DNA can be bound to proteins [43,44] and leukocyte activity influences the levels of cfDNA [42]. Intra-tumor heterogeneity and the amount of wild type tumor stroma DNA will have a great impact on the relative amount of mutant cfDNA [45]. Furthermore, age impacts the amount of cfDNA: Individuals of >90 years of age have elevated levels of cfDNA. Also, fragmented patterns and appearance of low molecular weight cfDNA was observed in the majority of the nonagenarians [46].

Secondly, technical aspects must be taken into account when assessing the quantity and quality of cfDNA. Levels of cfDNA are higher in plasma samples compared to serum samples because EDTA indirectly inhibits blood DNases [47]. Blood sample storage before spinning affects both the amounts and integrity of cfDNA [48] due to ongoing lysis of leukocytes before they are separated from the plasma by centrifugation. Furthermore, the wide variety in mutation detection techniques will impact sensitivity and specificity [38,49,50]. More recent studies report the use of targeted amplicon sequencing or massive parallel sequencing to cover a range of genes with great depth [51,52]. By using these recent approaches, the genomic heterogeneity as well as tumor burden can be revealed in more detail and with greater reliability than was possible even a few years ago.

Circulating cell-free mutant KRAS DNA in pancreatic cancer

Genomic mutations in the Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) gene are found in 80 to 90% of all pancreatic adenocarcinomas [53–55]. As a consequence, cfDNA from cancer patients should harbor these mutations and will be indicative of tumor-derived DNA. Data from numerous studies show the presence of mutant KRAS DNA in the circulation of patients with pancreatic cancer and are summarized in (Table 1). These results from a number of research groups show that the detection of circulating mutant KRAS DNA can be used to monitor pancreatic cancer progression. The diversity in PCR and sequencing techniques used across the studies, however, contributes to a varying detection rate of circulating mutant KRAS DNA. Also, the various technical aspects regarding cell free DNA isolation influence the quality of the mutant DNA measurements as discussed above. With respect to the specificity of mutant KRAS cfDNA for the detection of pancreatic cancer, the findings are mixed. Four studies did not detect circulating mutant KRAS DNA in patients with benign pancreatic pathology.

However, two studies described mutant KRAS in the circulation of 13% and 5% of patients (4 of 31 [56]; 2 of 37 [57]) with chronic pancreatitis. These patients then underwent surgery to remove the diseased organ. However, surgical pathology analysis of the tissues did not detect pancreatic cancer and after a follow-up of 6 to 36 months, none of the chronic pancreatitis patients with cfDNA KRAS mutations had developed pancreatic cancer. The transiently detected mutant KRAS DNA in the circulation likely originated from hyperplastic, mucous cells of chronically inflamed pancreas that had been removed and it thus appears that mutant KRAS cfDNA is not a reliable indicator of the presence of pancreatic cancer.

Circulating tumor DNA to evaluate tumor burden and therapeutic resistance

The majority of published studies on pancreatic cancer utilize mutant KRAS cfDNA as a diagnostic marker to confirm the presence of malignancy. When assessing alterations in circulating mutant KRAS as a marker of altered tumor size, one group found no correlation [57], whilst another group did find tumor size and abundance of circulating mutant KRAS cfDNA to be correlated [58]. Overall, the ratio of cancer cell to desmoplastic wildtype stroma will likely impact the abundance of circulating mutant KRAS and variability in the assessment of tumor/stroma ratio could explain the inconsistent findings across studies.

Furthermore, the abundance of mutant KRAS cfDNA could serve as a prognostic marker. Studies by Castells et al. and Chen et al. conclude that the abundance of circulating mutant KRAS DNA is associated with disease stage, metastasis and survival [57,59]. Uemura et al. found circulating mutant KRAS DNA in 5 out of 25 patients with stage III pancreatic cancer, in contrast to 25 out of 37 patients with stage IV disease [112]. Importantly, they showed that the survival of 30 patients with detectable KRAS mutations in circulating DNA is 6.3 months shorter than that of patients where only wildtype KRAS DNA was detected ($p<0.001$). After surgical removal of the tumor, cfDNA can serve as an indicator for the presence of residual cancer cells. Uemura et al. [112] showed that 5 out of 9 surgeries resulted in the disappearance of mutant KRAS cfDNA. Additionally, Yamada et al. [58] found that surgery and/or chemoradiation treatment resulted in the disappearance of mutant KRAS from plasma DNA in 12 out of 15 patients. A recent study by Newman et al. [38] in lung cancer patients they used a more sensitive DNA detection method for mutant cfDNA levels and found a correlation with tumor volumes during chemotherapy and surgery and with CT and PET scan data. Thus, technical aspects of sample handling, tumor size measurements and DNA measurement may account for the inconsistencies seen in the earlier pancreatic cancer studies.

In contrast to blood samples, small tissue biopsies will provide limited and possibly erroneous genetic characterization due to the spatial and temporal intra-tumor heterogeneity [60]. The mutation pattern in a tumor can be indeed derived from deep sequencing analysis of cfDNA [51,52] and cfDNA can be used to reveal clonal evolution due to drug treatments [51,61]. During the course of treatment, the somatic alterations of resistant clones can be accurately followed using cfDNA, which can be applied to tailor the therapy to a specific patient. No such studies have been published in pancreatic cancers. A study by Diaz et al. on

mutant KRAS cfDNA in colon cancer patients could serve as a paradigm [62]: They found that 9 of 24 patients whose colorectal tumors were initially KRAS wildtype, developed detectable mutations in KRAS in their cfDNA after treatment with an EGFR antibody. The appearance of these mutations was very consistent, occurring between 5 and 6 months following treatment. This suggests that the emergence of KRAS mutant clones was due to the selection for resistance to EGFR blockade. These findings suggest potential utility also for pancreatic cancer monitoring via cfDNA analysis.

Circulating miRs

MiR are non-coding ribonucleic acids of ~22 nucleotides in length, which regulate mRNA stability and translation of known protein coding genes [63]. MiR genes are transcribed from individual genetic loci [64] and show complementary binding to 3' or 5'-untranslated regions (UTR) of the targeted mRNA or to the open reading frames. Via this interaction miR can lead to mRNA degradation or inhibition of protein translation [65,66]. Due to the relatively short seed sequence a single miR may target hundreds of mRNAs. Thus miR are involved in virtually all physiological and pathological processes and found altered in every type of cancer studied to date [67].

Cells export miR -containing apoptotic bodies, shedding vesicles, and exosomes into the bloodstream [68–71]. Microvesicles are impermeable to RNases, which explains the remarkable stability of extracellular miR. Additionally, a majority of extracellular miR in plasma or serum are membrane vesicle-free, but associated with 1 of 4 proteins of the Argonaute (AGO) family [72]. The remarkable stability of AGO2 protein explains the stability of associated miR even in nuclease- and protease-rich environments [71]. Data from a number of laboratories suggest that different RNA species can be specifically packaged into microvesicles by active sorting mechanisms which have not been fully elucidated [73]. Blood cells are major contributors to extracellular miR content in the circulation [74]. Different organs contribute to circulating miR because tissue-specific miR such as miR -122 (liver), miR -133a (muscle), miR -208a (heart), and miR -124 (brain) have been consistently detected in plasma samples [75–77]. Tumors release miR into the bloodstream and cancer tissue-specific miR have been found in the circulation at different stages of the disease (for a recent review of circulating miR in cancer see Schwarzenbach et al. [78]).

Circulating miR entrapped within microvesicles can be transferred to recipient cells as signaling molecules and alter gene expression in target cells [73]. Once in the circulation, miR can function as hormones and a given miR may function as an oncogene or a tumor-suppressor gene depending on the cellular context and the targeted organ [79]. The concentration of miR in the bloodstream reflects alterations in the homeostasis of the entire organism and a panel of miR can easily be quantitated by quantitative PCR [80]. Findings from such studies have established the basis for circulating miR as disease biomarkers.

Factors impacting circulating miR levels

Cell free circulating miR can be obtained by using plasma that is collected in EDTA tubes or serum collected in the absence of anticoagulants. Both can produce satisfactory results [81]. However, plasma collection using heparin as an anticoagulant interferes with the

quantitation of miR species using PCR-based approaches [82]. Even though this inhibition can be alleviated by digesting heparin with lyase heparinase I prior to reverse transcription, it leads to lower efficiency and higher costs. Blood samples have to be centrifuged promptly to separate the cell-free phase and prevent hemolysis. Excessive concentrations of miR-16 and miR-451 can be indicative of hemolysis [83]. Before extraction of circulating miR, cells must be removed by centrifugation at about $1,200 \times g$. Furthermore, dead cells, apoptotic bodies, large shed vesicles, and cell debris can be removed by high-speed centrifugation $14,000$ to $16,000 \times g$ [84]. To monitor the efficiency of miR isolation, synthetic miR controls (SYNTH) can be used by spiking samples. When extracting miRs from blood samples, endogenous inhibitors of polymerases must be removed as much as possible: Trizol reagents are an example of reagents that will serve this purpose [85]. Polymerase inhibitors may include IgGs, hemoglobin or lactoferrin. Measuring miR levels by Taqman qPCR has become a standard method. In the case of very low RNA yields, the use of Locked Nucleic Acid-enhanced primers improves the sensitivity of the qPCR [86]. Furthermore, mutated Taq polymerases are less sensitive to blood-borne inhibitors. A Taq polymerase mixture of normal and Hemo KlenTaq improves sensitivity up to 30-fold as reported by Kim et al. [81]. To account for different extraction efficiency, different approaches have been used to compare miR concentrations across samples. If a large number of miR is detected in a single sample, the mean (or median) abundance can be used for normalization. There are many reports on using levels of endogenous miRs as internal reference control [87–89]. However, caution has to be taken before using this approach, since biological and technical processes can greatly influence the abundance of the miRs.

Circulating miR in pancreatic cancer

A wealth of information indicating the potential use of circulating miR for pancreatic cancer screening has emerged over the past years (Table 2). In 2014, Ganepola et al. performed a non-biased screening method to develop a panel of blood based diagnostic biomarkers consisting of circulating miR for the detection of pancreatic cancer at an early stage. They compared 8 early stage pancreatic cancer patients to 11 healthy controls and performed a high throughput screening using hybridization microarray analysis [90]. After validation studies, 3 miR, miR -22, miR -642b and miR-885-5p were confirmed to show consistent expression between microarray and qPCR. These 3 miR were then validated and evaluated as a diagnostic panel with a new cohort of patients and controls and found to yield 91% sensitivity and 91% specificity. The CA19-9 marker showed 73% sensitivity and 100% specificity.

In a large study by Kojima et al. 571 serum samples obtained from healthy subjects, patients with pancreatic, biliary-tract, or other digestive cancers and patients with non-malignant abnormalities in the pancreas or biliary tract were analyzed. Expression levels of miRs were obtained by a 3D-Gene microarray. They found that a combination of 8 miRs (miR-125a-3p, miR-4294, miR-4476, miR-4530, miR-6075, miR-6799-5p, miR-6836-3p and miR-6880-5p) achieved the best discriminant performance. The sensitivity was 80.3%, specificity 97.6% and accuracy 91.6% in detecting pancreatobiliary cancers contrasted with healthy controls, non-malignant abnormalities or other types of cancers [91].

There are some reports that extend beyond cancer related miRs, which show that similar miRs are indicators of benign pancreas pathology. Chronic pancreatitis is a risk factor for pancreatic cancer and shares clinical symptoms with pancreatic cancer. For example, circulating miR-155, 181a, 181b, 196a, 200a, 200b, and 212 were reported as significantly upregulated in patients with chronic pancreatitis versus healthy controls [17,92–94]. Li et al. showed that levels of serum miR-200a and miR-200b are similar in pancreatic cancer and pancreatitis patients, although they are increased compared to healthy controls [94]. Interestingly the miR-200 family can inhibit epithelial to mesenchymal transition and may thus have a direct signaling role [95]. In conclusion, circulating miRs can indicate the presence of chronic inflammatory and potentially premalignant processes in the pancreas.

Circulating miRs reflect disease state, outcome and drug response

Pancreatic cancer is a systemic disease that encompasses altered glucose metabolism [96,97], stroma remodeling [98], and bone marrow derived cellular activity [99,100]. The involvement of circulating miRs in such processes makes them excellent candidates to reflect the systemic impact of the disease and may provide additional molecular markers derived from other tissues impacted by the disease, e.g. the bone marrow. Ali et al. found that a high level of plasma miR-21 was correlated with worse survival, and the expression of let-7 was inversely correlated with survival [101]. In addition, Abue et al. tested the levels of miR-483-3p and miR-21 in plasma of 32 pancreatic cancer patients, 12 patients with intraductal papillary mucinous neoplasm (IPMN) and 30 healthy controls and reported that plasma miR-21 levels were associated with advanced cancer stage, lymph node and liver metastasis, and shorter survival [102].

Surgical removal of pancreatic cancer lowered circulating miR-221 in 8 patients [103] and miR-18a dropped significantly after surgical removal of pancreatic cancer in 9 patients [104]. Strikingly, at the time of tumor recurrence in one patient, the levels of circulating miR-18a had increased again despite the lack of any elevation of the conventional serum tumor marker CA-19-9. Additionally, circulating miR-483-3p levels decreased after surgery in 2 pancreatic cancer patients [102] and Li et al. [105] report that higher miR-1290 levels predicted poorer outcome among patients undergoing pancreaticoduodenectomy.

Drug therapy impacts many organ systems. MiR concentrations in the circulation could serve as easily accessible markers of treatment efficacy and even indicate pathways altered by a given treatment. Wang et al. showed that drug induced liver injury can be indicated by a dramatic increase in plasma miR-122 and decrease in miR-170. The circulating miRs showed to be more sensitive markers than alanine aminotransferase for liver injury [106]. We studied circulating miRs before and after Gemcitabine treatment in healthy and pancreatic cancer-bearing mice [107]. MiR-10, miR-21 and miR-155 serum levels decreased significantly after Gemcitabine treatment in both healthy mice and in KrasG12D driven pancreatic cancer mice. Interestingly, serum miR-155 concentrations in mice with pancreatic cancer were significantly lower than in control mice upon Gemcitabine treatment suggesting miR-155 as a potential indicator of tumor specific effects of the treatment.

In a report by Shivapurkar et al. we showed that circulating miR-296 is lost during tumor progression and correlates with metastatic disease in colorectal cancer [108]. Patients with metastatic colon cancer were treated with a multi-targeted receptor tyrosine kinase inhibitor, Sunitinib, and the anti-metabolite Capecitabine. Circulating miRs were analyzed from 7 pre- and matching post-treatment serum samples. 3 patients had decreased miR-296 at 4 weeks post treatment. 4 patients had an increase in the level of miR-296 during that time period. Compared to patients with longer survival and better clinical outcome, patients with shorter survival and poor clinical outcome exhibited a decrease in the level of miR-296 at 4 weeks compared to baseline. The loss of miR-296 may be one of the mechanisms for primary resistance of colorectal cancer to chemotherapy and this could be translated to studies in pancreatic cancer patients using similar drug regimen.

Conclusions

“Liquid biopsies”, a recently adopted term for blood based molecular analyses in cancer patient diagnostics can provide innovative monitoring of the evolution of the disease and the response to treatment. Both circulating microRNA and cancer cell derived mutant DNA have been tested as potential surrogates or complements for direct tissue biopsies. A major strength of liquid biopsies is the possibility to compare serial samples from the same patient and thus generate a molecular readout of disease progression and therapy response or resistance in real time. Also the blood draw is minimally invasive and provides bio specimen of comparable composition from a homogeneous compartment, i.e. the blood stream.

Mutant cfDNA and circulating miRs provide distinct and complementary information: Alterations in cfDNA mutation patterns originate from treatment resistant cancer cell subpopulations and their emergence could prompt changes in treatment. On the other hand, altered patterns of circulating miR expression indicate a change in the steady state of the whole organism that carries the disease in response to treatment as well as disease response or progression. Thus, we propose to use a combination analysis of circulating mutant DNA and microRNAs to monitor treatment responses in patients with pancreatic cancer (Figure 1). However, sensitive detection methods and extensive analyses are needed to uncover indicators of diseased organs in a background of circulating nucleic acids from healthy organs. Figure 1 illustrates this challenge and the opportunity to provide deeper insights into the molecular alterations driving pancreatic cancers.

Acknowledgments

We would like to thank Dr. Narayan Shivapurkar who shared his experience in isolating and measuring circulating miRs, Dr. Ivana Peran for her thoughts on the use of circulating miRs as drug response markers, Jeroen Versteeg, for discussions on circulating miRs in patients with pancreatic cancer and Dr. Niels Kok for discussions on clinical utility of circulating nucleic acids in cancer patients. Work was supported in part by NIH/NCI grants R01 CA71508 (AW), R33 CA177466 (EEV, AW) and P30 CA51008 (AW) as well as the Ruesch Center at Georgetown University (AW).

References

1. Ma J, Siegel R, Jemal A. Pancreatic cancer death rates by race among US men and women, 1970–2009. *J Natl Cancer Inst.* 2013; 105:1694–1700. [PubMed: 24203988]

2. Seufferlein T, Porzner M, Heinemann V, Tannapfel A, Stuschke M, et al. Ductal pancreatic adenocarcinoma. *Dtsch Arztebl Int.* 2014; 111:396–402. [PubMed: 24980565]
3. Heinemann V, Haas M, Boeck S. Systemic treatment of advanced pancreatic cancer. *Cancer Treat Rev.* 2012; 38:843–853. [PubMed: 22226241]
4. Ueno H, Ioka T, Ikeda M, Ohkawa S, Yanagimoto H, et al. Randomized phase III study of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer in Japan and Taiwan: GEST study. *J Clin Oncol.* 2013; 31:1640–1648. [PubMed: 23547081]
5. Marsh RW, Talamonti MS, Katz MH, Herman JM. Pancreatic cancer and FOLFIRINOX: a new era and new questions. *Cancer Med.* 2015
6. Liu L, Xu H, Wang W, Wu C, Chen Y, et al. A preoperative serum signature of CEA+/CA125+/CA19-9 ≥1000 U/mL indicates poor outcome to pancreatectomy for pancreatic cancer. *Int J Cancer.* 2015; 136:2216–2227. [PubMed: 25273947]
7. Brown EG, Canter RJ, Bold RJ. Preoperative CA 19-9 kinetics as a prognostic variable in radiographically resectable pancreatic adenocarcinoma. *J Surg Oncol.* 2015; 111:293–298. [PubMed: 25330934]
8. E Nakeeb A, E Shobary M, E Dosoky M, Nabeh A, E Sorogy M, et al. Prognostic factors affecting survival after pancreaticoduodenectomy for pancreatic adenocarcinoma (single center experience). *Hepatogastroenterology.* 2014; 61:1426–1438.
9. Leon SA, Ehrlich GE, Shapiro B, Labbate VA. Free DNA in the serum of rheumatoid arthritis patients. *J Rheumatol.* 1977; 4:139–143. [PubMed: 881690]
10. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res.* 1977; 37:646–650. [PubMed: 837366]
11. Shapiro B, Chakrabarty M, Cohn EM, Leon SA. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. *Cancer.* 1983; 51:2116–2120. [PubMed: 6188527]
12. MANDEL P, METAIS P. Not Available. *C R Seances Soc Biol Fil.* 1948; 142:241–243. [PubMed: 18875018]
13. Anker P, Lyautey J, Lederrey C, Stroun M. Circulating nucleic acids in plasma or serum. *Clin Chim Acta.* 2001; 313:143–146. [PubMed: 11694252]
14. McIlroy DJ, Bigland M, White AE, Hardy BM, Lott N, et al. Cell necrosis-independent sustained mitochondrial and nuclear DNA release following trauma surgery. *J Trauma Acute Care Surg.* 2015; 78:282–288. [PubMed: 25602756]
15. Cui M, Fan M, Jing R, Wang H, Qin J, et al. Cell-Free circulating DNA: a new biomarker for the acute coronary syndrome. *Cardiology.* 2013; 124:76–84. [PubMed: 23363853]
16. Rainer TH, Lam NY, Man CY, Chiu RW, Woo KS, et al. Plasma beta-globin DNA as a prognostic marker in chest pain patients. *Clin Chim Acta.* 2006; 368:110–113. [PubMed: 16480967]
17. Shimony A, Zahger D, Gilutz H, Goldstein H, Orlov G, et al. Cell free DNA detected by a novel method in acute ST-elevation myocardial infarction patients. *Acute Card Care.* 2010; 12:109–111. [PubMed: 20712451]
18. Bagul A, Pushpakom S, Boylan J, Newman W, Siriwardena AK. Quantitative analysis of plasma DNA in severe acute pancreatitis. *JOP.* 2006; 7:602–607. [PubMed: 17095839]
19. Nakahira K, Kyung SY2, Rogers AJ3, Gazourian L4, Youn S4, et al. Circulating mitochondrial DNA in patients in the ICU as a marker of mortality: derivation and validation. *PLoS Med.* 2013; 10:e1001577. [PubMed: 24391478]
20. Rainer TH, Lam NY. Circulating nucleic acids and critical illness. *Ann N Y Acad Sci.* 2006; 1075:271–277. [PubMed: 17108220]
21. Fatouros IG, Destouni A, Margonis K, Jamurtas AZ, Vrettou C, et al. Cell-free plasma DNA as a novel marker of aseptic inflammation severity related to exercise overtraining. *Clin Chem.* 2006; 52:1820–1824. [PubMed: 16840584]
22. Chan KC, Zhang J, Hui AB, Wong N, Lau TK, et al. Size distributions of maternal and fetal DNA in maternal plasma. *Clin Chem.* 2004; 50:88–92. [PubMed: 14709639]
23. Chiu RW, Lo YM. Clinical applications of maternal plasma fetal DNA analysis: translating the fruits of 15 years of research. *Clin Chem Lab Med.* 2013; 51:197–204. [PubMed: 23072857]

24. Lo YM, Chan KC, Sun H, Chen EZ, Jiang P, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci Transl Med*. 2010; 2:61ra91.
25. Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. *Clin Chim Acta*. 2001; 313:139–142. [PubMed: 11694251]
26. van der Vaart M, Pretorius PJ. Circulating DNA. Its origin and fluctuation. *Ann N Y Acad Sci*. 2008; 1137:18–26. [PubMed: 18837919]
27. Nagata S, Nagase H, Kawane K, Mukae N, Fukuyama H. Degradation of chromosomal DNA during apoptosis. *Cell Death Differ*. 2003; 10:108–116. [PubMed: 12655299]
28. Holdenrieder S, Stieber P. Apoptotic markers in cancer. *Clin Biochem*. 2004; 37:605–617. [PubMed: 15234242]
29. Atamaniuk J, Ruzicka K, Stuhlmeier KM, Karimi A, Eigner M, et al. Cell-free plasma DNA: a marker for apoptosis during hemodialysis. *Clin Chem*. 2006; 52:523–526. [PubMed: 16510434]
30. Giacona MB, Ruben GC, Iczkowski KA, Roos TB, Porter DM, et al. Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. *Pancreas*. 1998; 17:89–97. [PubMed: 9667526]
31. Holdenrieder S, Stieber P, von Pawel J, Raith H, Nagel D, et al. Circulating nucleosomes predict the response to chemotherapy in patients with advanced non-small cell lung cancer. *Clin Cancer Res*. 2004; 10:5981–5987. [PubMed: 15447981]
32. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res*. 2001; 61:1659–1665. [PubMed: 11245480]
33. Anker P, Stroun M, Maurice PA. Spontaneous release of DNA by human blood lymphocytes as shown in an in vitro system. *Cancer Res*. 1975; 35:2375–2382. [PubMed: 1149042]
34. Rogers JC, Boldt D, Kornfeld S, Skinner A, Valeri CR. Excretion of deoxyribonucleic acid by lymphocytes stimulated with phytohemagglutinin or antigen. *Proc Natl Acad Sci U S A*. 1972; 69:1685–1689. [PubMed: 4505646]
35. Abolhassani M, Tillotson J, Chiao J. Characterization of the release of DNA by a human leukemia-cell line hl-60. *Int J Oncol*. 1994; 4:417–421. [PubMed: 21566940]
36. Singh N, Gupta S, Pandey RM, Chauhan SS, Saraya A. High levels of cell-free circulating nucleic acids in pancreatic cancer are associated with vascular encasement, metastasis and poor survival. *Cancer Invest*. 2015; 33:78–85. [PubMed: 25647443]
37. Bettgeowda C, Sausen M, Leary RJ, Kinde I, Wang Y, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014; 6:224ra24.
38. Newman AM, Bratman SV2, To J3, Wynne JF3, Eclow NC3, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat Med*. 2014; 20:548–554. [PubMed: 24705333]
39. Sikora K, Bedin C, Vicentini C, Malpeli G, D'Angelo E, et al. Evaluation of cell-free DNA as a biomarker for pancreatic malignancies. *Int J Biol Markers*. 2015; 30:e136–141. [PubMed: 24832178]
40. Su YH, Wang M, Brenner DE, Ng A, Melkonyan H, et al. Human urine contains small, 150 to 250 nucleotide-sized, soluble DNA derived from the circulation and may be useful in the detection of colorectal cancer. *J Mol Diagn*. 2004; 6:101–107. [PubMed: 15096565]
41. Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, et al. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet*. 1999; 64:218–224. [PubMed: 9915961]
42. Tamkovich SN, Cherepanova AV, Kolesnikova EV, Rykova EY, Pyshnyi DV, et al. Circulating DNA and DNase activity in human blood. *Ann N Y Acad Sci*. 2006; 1075:191–196. [PubMed: 17108211]
43. Gerasimova YV, Alekseyeva IV, Bogdanova TG, Erchenko IA, Kudryashova NV, et al. Affinity separation of polyribonucleotide-binding human blood proteins. *Bioorg Med Chem Lett*. 2006; 16:5526–5529. [PubMed: 16934459]
44. Chelobanov BP, Laktionov PP, Vlasov VV. Proteins involved in binding and cellular uptake of nucleic acids. *Biochemistry (Mosc)*. 2006; 71:583–596. [PubMed: 16827649]

45. Lennerz JK, Stenzinger A2. Allelic Ratio of KRAS Mutations in Pancreatic Cancer. *Oncologist*. 2015; 20:e8–9. [PubMed: 25777349]
46. Jylhävä J, Kotipelto T, Raitala A, Jylhä M, Hervonen A, et al. Aging is associated with quantitative and qualitative changes in circulating cell-free DNA: the Vitality 90+ study. *Mech Ageing Dev*. 2011; 132:20–26. [PubMed: 21078336]
47. Barra GB, Santa Rita TH, Vasques JA, Chianca CF, Nery LF, et al. EDTA-mediated inhibition of DNases protects circulating cell-free DNA from ex vivo degradation in blood samples. *Clin Biochem*. 2015
48. Wong D, Moturi S, Angkachatchai V, Mueller R, DeSantis G, et al. Optimizing blood collection, transport and storage conditions for cell free DNA increases access to prenatal testing. *Clin Biochem*. 2013; 46:1099–1104. [PubMed: 23643886]
49. Luke JJ, Oxnard GR, Paweletz CP, Camidge D, Heymach JV, et al. Realizing the potential of plasma genotyping in an age of genotype-directed therapies. *J Natl Cancer Inst*. 2014; 106
50. Wu J, Zhou Y, Zhang CY, Song BB, Wang BL, et al. Co-amplification at lower denaturation-temperature PCR combined with unlabeled-probe high-resolution melting to detect KRAS codon 12 and 13 mutations in plasmacirculating DNA of pancreatic adenocarcinoma cases. *Asian Pac J Cancer Prev*. 2014; 15:10647–10652. [PubMed: 25605154]
51. De Mattos-Arruda L, Weigelt B, Cortes J, Won HH, Ng CK, et al. Capturing intra-tumor genetic heterogeneity by de novo mutation profiling of circulating cell-free tumor DNA: a proof-of-principle. *Ann Oncol*. 2014; 25:1729–1735. [PubMed: 25009010]
52. Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DW, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci Transl Med*. 2012; 4:136ra68.
53. Grünewald K, Lyons J, Fröhlich A, Feichtinger H, Weger RA, et al. High frequency of Ki-ras codon 12 mutations in pancreatic adenocarcinomas. *Int J Cancer*. 1989; 43:1037–1041. [PubMed: 2659539]
54. Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, et al. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell*. 1988; 53:549–554. [PubMed: 2453289]
55. Smit VT, Boot AJ, Smits AM, Fleuren GJ, Cornelisse CJ, et al. KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas. *Nucleic Acids Res*. 1988; 16:7773–7782. [PubMed: 3047672]
56. Maire F, Micard S, Hammel P, Voitot H, Lévy P, et al. Differential diagnosis between chronic pancreatitis and pancreatic cancer: value of the detection of KRAS2 mutations in circulating DNA. *Br J Cancer*. 2002; 87:551–554. [PubMed: 12189555]
57. Castells A, Puig P, Móra J, Boadas J, Boix L, et al. K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. *J Clin Oncol*. 1999; 17:578–584. [PubMed: 10080602]
58. Yamada T, Nakamori S, Ohzato H, Oshima S, Aoki T, et al. Detection of K-ras gene mutations in plasma DNA of patients with pancreatic adenocarcinoma: correlation with clinicopathological features. *Clin Cancer Res*. 1998; 4:1527–1532. [PubMed: 9626473]
59. Chen H, Tu H, Meng ZQ, Chen Z, Wang P, et al. K-ras mutational status predicts poor prognosis in unresectable pancreatic cancer. *Eur J Surg Oncol*. 2010; 36:657–662. [PubMed: 20542658]
60. Jamal-Hanjani M, Quezada SA2, Larkin J3, Swanton C4. Translational implications of tumor heterogeneity. *Clin Cancer Res*. 2015; 21:1258–1266. [PubMed: 25770293]
61. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature*. 2013; 497:108–112. [PubMed: 23563269]
62. Diaz LA Jr, Williams RT, Wu J, Kinde I, Hecht JR, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature*. 2012; 486:537–540. [PubMed: 22722843]
63. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005; 120:15–20. [PubMed: 15652477]

64. Lee Y, Kim M, Han J, Yeom KH, Lee S, et al. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J*. 2004; 23:4051–4060. [PubMed: 15372072]
65. Lytle JR, Yario TA, Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc Natl Acad Sci U S A*. 2007; 104:9667–9672. [PubMed: 17535905]
66. Place RF, Li LC, Pookot D, Noonan EJ, Dahiya R. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A*. 2008; 105:1608–1613. [PubMed: 18227514]
67. Rosenfeld N, Aharonov R, Meiri E, Rosenwald S, Spector Y, et al. MicroRNAs accurately identify cancer tissue origin. *Nat Biotechnol*. 2008; 26:462–469. [PubMed: 18362881]
68. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007; 9:654–659. [PubMed: 17486113]
69. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, et al. Detection of microRNA expression in human peripheral blood microvesicles. *PLoS One*. 2008; 3:e3694. [PubMed: 19002258]
70. Zerneck A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, et al. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. *Sci Signal*. 2009; 2:ra81. [PubMed: 19996457]
71. Turchinovich A, Weiz L, Langhein A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res*. 2011; 39:7223–7233. [PubMed: 21609964]
72. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A*. 2011; 108:5003–5008. [PubMed: 21383194]
73. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, et al. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem*. 2010; 285:17442–17452. [PubMed: 20353945]
74. Pritchard CC, Kroh E, Wood B, Arroyo JD, Dougherty KJ, et al. Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer Prev Res (Phila)*. 2012; 5:492–497. [PubMed: 22158052]
75. Lewis AP, Jopling CL. Regulation and biological function of the liver-specific miR-122. *Biochem Soc Trans*. 2010; 38:1553–1557. [PubMed: 21118125]
76. Laterza OF, Lim L, Garrett-Engele PW, Vlasakova K, Muniappa N, et al. Plasma MicroRNAs as sensitive and specific biomarkers of tissue injury. *Clin Chem*. 2009; 55:1977–1983. [PubMed: 19745058]
77. Corsten MF, Dennert R, Jochems S, Kuznetsova T, Devaux Y, et al. Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. *Circ Cardiovasc Genet*. 2010; 3:499–506. [PubMed: 20921333]
78. Schwarzenbach H, Nishida N, Calin GA, Pantel K. Clinical relevance of circulating cell-free microRNAs in cancer. *Nat Rev Clin Oncol*. 2014; 11:145–156. [PubMed: 24492836]
79. Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, et al. MicroRNAs in body fluids--the mix of hormones and biomarkers. *Nat Rev Clin Oncol*. 2011; 8:467–477. [PubMed: 21647195]
80. Hastings ML, Palma J, Duelli DM. Sensitive PCR-based quantitation of cell-free circulating microRNAs. *Methods*. 2012; 58:144–150. [PubMed: 22884953]
81. Kim DJ, Linnstaedt S, Palma J, Park JC, Ntrivalas E, et al. Plasma components affect accuracy of circulating cancer-related microRNA quantitation. *J Mol Diagn*. 2012; 14:71–80. [PubMed: 22154918]
82. Johnson ML, Navanukraw C, Grazul-Bilska AT, Reynolds LP, Redmer DA. Heparinase treatment of RNA before quantitative real-time RT-PCR. *Biotechniques*. 2003; 35:1140–1142. 1144. [PubMed: 14682046]
83. Kirschner MB, Kao SC, Edelman JJ, Armstrong NJ, Vallety MP, et al. Haemolysis during sample preparation alters microRNA content of plasma. *PLoS One*. 2011; 6:e24145. [PubMed: 21909417]
84. Turchinovich A, Weiz L, Burwinkel B. Extracellular miR: the mystery of their origin and function. *Trends Biochem Sci*. 2012; 37:460–465. [PubMed: 22944280]

85. Rio DC, Ares M, Hannon GJ, Nilsen TW. Purification of RNA using TRIzol (TRI reagent). Cold Spring Harb Protoc. 2010; 2010:prot5439.
86. Andreasen D, Fog JU, Biggs W, Salomon J, Dahlsveen IK, et al. Improved microRNA quantification in total RNA from clinical samples. Methods. 2010; 50:S6–9. [PubMed: 20215018]
87. Zhu HT, Dong QZ, Wang G, Zhou HJ, Ren N, et al. Identification of suitable reference genes for qRT-PCR analysis of circulating microRNAs in hepatitis B virus-infected patients. Mol Biotechnol. 2012; 50:49–56. [PubMed: 21567136]
88. Appaiah HN, Goswami CP, Mina LA, Badve S, Sledge GW Jr, et al. Persistent upregulation of U6:SNORD44 small RNA ratio in the serum of breast cancer patients. Breast Cancer Res. 2011; 13:R86. [PubMed: 21914171]
89. Hu Z, Dong J, Wang LE, Ma H, Liu J, et al. Serum microRNA profiling and breast cancer risk: the use of miR-484/191 as endogenous controls. Carcinogenesis. 2012; 33:828–834. [PubMed: 22298638]
90. Ganepola GA, Rutledge JR, Suman P, Yiengpruksawan A, Chang DH. Novel blood-based microRNA biomarker panel for early diagnosis of pancreatic cancer. World J Gastrointest Oncol. 2014; 6:22–33. [PubMed: 24578785]
91. Kojima M, Sudo H, Kawauchi J, Takizawa S, Kondou S, et al. MicroRNA markers for the diagnosis of pancreatic and biliary-tract cancers. PLoS One. 2015; 10:e0118220. [PubMed: 25706130]
92. Cote GA, Gore AJ, McElyea SD, Heathers LE, Xu H, et al. A pilot study to develop a diagnostic test for pancreatic ductal adenocarcinoma based on differential expression of select miRNA in plasma and bile. Am J Gastroenterol. 2014; 109:1942–1952. [PubMed: 25350767]
93. Liu J, Gao J, Du Y, Li Z, Ren Y, et al. Combination of plasma microRNAs with serum CA19-9 for early detection of pancreatic cancer. Int J Cancer. 2012; 131:683–691. [PubMed: 21913185]
94. Li A, Omura N, Hong SM, Vincent A, Walter K, et al. Pancreatic cancers epigenetically silence SIP1 and hypomethylate and overexpress miR-200a/200b in association with elevated circulating miR-200a and miR-200b levels. Cancer Res. 2010; 70:5226–5237. [PubMed: 20551052]
95. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008; 10:593–601. [PubMed: 18376396]
96. Yu M, Zhou Q, Zhou Y, Fu Z, Tan L, et al. Metabolic phenotypes in pancreatic cancer. PLoS One. 2015; 10:e0115153. [PubMed: 25719198]
97. Bryant KL, Mancias JD, Kimmelman AC, Der CJ. KRAS: feeding pancreatic cancer proliferation. Trends Biochem Sci. 2014; 39:91–100. [PubMed: 24388967]
98. Whatcott CJ, Diep CH, Jiang P, Watanabe A, LoBello J, et al. Desmoplasia in primary tumors and metastatic lesions of pancreatic cancer. Clin Cancer Res. 2015
99. Greten TF. Myeloid-derived suppressor cells in pancreatic cancer: more than a hidden barrier for antitumour immunity? Gut. 2014; 63:1690–1691. [PubMed: 24633728]
100. Mielgo A, Schmid MC. Impact of tumour associated macrophages in pancreatic cancer. BMB Rep. 2013; 46:131–138. [PubMed: 23527856]
101. Ali S, Almhanna K, Chen W, Philip PA, Sarkar FH. Differentially expressed miR in the plasma may provide a molecular signature for aggressive pancreatic cancer. Am J Transl Res. 2010; 3:28–47. [PubMed: 21139804]
102. Abue M, Yokoyama M, Shibuya R, Tamai K, Yamaguchi K, et al. Circulating miR-483-3p and miR-21 is highly expressed in plasma of pancreatic cancer. Int J Oncol. 2015; 46:539–547. [PubMed: 25384963]
103. Kawaguchi T, Komatsu S, Ichikawa D, Morimura R, Tsujiura M, et al. Clinical impact of circulating miR-221 in plasma of patients with pancreatic cancer. Br J Cancer. 2013; 108:361–369. [PubMed: 23329235]
104. Morimura R, Komatsu S, Ichikawa D, Takeshita H, Tsujiura M, et al. Novel diagnostic value of circulating miR-18a in plasma of patients with pancreatic cancer. Br J Cancer. 2011; 105:1733–1740. [PubMed: 22045190]

105. Li A, Yu J, Kim H, Wolfgang CL, Canto MI, et al. MicroRNA array analysis finds elevated serum miR-1290 accurately distinguishes patients with low-stage pancreatic cancer from healthy and disease controls. *Clin Cancer Res*. 2013; 19:3600–3610. [PubMed: 23697990]
106. Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proc Natl Acad Sci U S A*. 2009; 106:4402–4407. [PubMed: 19246379]
107. LaConti JJ, Shivapurkar N, Preet A, Deslattes Mays A, Peran I, et al. Tissue and serum microRNAs in the Kras(G12D) transgenic animal model and in patients with pancreatic cancer. *PLoS One*. 2011; 6:e20687. [PubMed: 21738581]
108. Shivapurkar N, Mikhail S, Navarro R, Bai W, Marshall J, et al. Decrease in blood miR-296 predicts chemotherapy resistance and poor clinical outcome in patients receiving systemic chemotherapy for metastatic colon cancer. *Int J Colorectal Dis*. 2013; 28:887. [PubMed: 22892985]
109. Mulcahy HE, Lyautey J, Lederrey C, qi Chen X, Anker P, et al. A prospective study of K-ras mutations in the plasma of pancreatic cancer patients. *Clin Cancer Res*. 1998; 4:271–275. [PubMed: 9516910]
110. Dianxu F, Shengdao Z, Tianquan H, Yu J, Ruoqing L, et al. A prospective study of detection of pancreatic carcinoma by combined plasma K-ras mutations and serum CA19-9 analysis. *Pancreas*. 2002; 25:336–341. [PubMed: 12409826]
111. Däbritz J, Preston R, Hänfler J, Oettle H. Follow-up study of K-ras mutations in the plasma of patients with pancreatic cancer: correlation with clinical features and carbohydrate antigen 19-9. *Pancreas*. 2009; 38:534–541. [PubMed: 19295453]
112. Uemura T, Hibi K, Kaneko T, Takeda S, Inoue S, et al. Detection of K-ras mutations in the plasma DNA of pancreatic cancer patients. *J Gastroenterol*. 2004; 39:56–60. [PubMed: 14767735]
113. Wang J, Chen J, Chang P, LeBlanc A, Li D, et al. MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease. *Cancer Prev Res (Phila)*. 2009; 2:807–813. [PubMed: 19723895]
114. Ho AS, Huang X, Cao H, Christman-Skieller C, Bennewith K, et al. Circulating miR-210 as a novel hypoxia marker in pancreatic cancer. *Transl Oncol*. 2010; 3:109. [PubMed: 20360935]
115. Chen Q, Yang L, Xiao Y, Zhu J, Li Z. Circulating microRNA-182 in plasma and its potential diagnostic and prognostic value for pancreatic cancer. *Med Oncol*. 2014; 31:225. [PubMed: 25326859]
116. Lin MS, Chen WC, Huang JX, Gao HJ, Sheng HH. Aberrant expression of microRNAs in serum may identify individuals with pancreatic cancer. *Int J Clin Exp Med*. 2014; 7:5226–5234. [PubMed: 25664025]

Figure 1.

Release of cell free DNA and miR into the circulation. Healthy organs e.g. brain, lung, heart, kidney, liver, bone marrow shed miR and wild type DNA into the bloodstream as indicated by the dotted arrows. The pancreatic cancer (enlarged area) is depicted as cancer cells (colored) and stromal cells (grey). Tumor-derived mutated DNA originates from the heterogeneous cancer cell population and is shed into the circulation as cell-free (cf) DNA or from apoptotic cancer cells that entered the circulation. MicroRNAs derived from normal and from tumor tissues reflect the composite of signaling in cancer cells, stroma and normal tissues. Double helices indicate cfDNA, short, single-stranded nucleic acids in different colors indicate miR.

Table 1
Summary of published reports on circulating mutant KRAS DNA in patients with pancreatic cancer.

Number of patients with pancreatic cancer patients with circulating mutant KRAS DNA	Detection method	Pancreatic cancer stage	Number of patients without pancreatic cancer with circulating mutant KRAS DNA remarks	Remarks	Reference
17/21 (81%)	PCR-RFLP followed by sequencing	unresectable disease	0/5 healthy 0/3 CP	2 patients had circulating mutant KRAS without detected mutant KRAS in the cancer tissue	Mulcahy et al. 1998 [109]
29/41 (71%)	PCR-RFLP followed by gel electrophoresis	stages I-IV	0/21 healthy 0/4 CP 0/3 cyst adenoma	no relationship with tumor stage	Dainxu et al. 2002 [110]
9/15 (60%)	MASA-PCR followed by gel electrophoresis	stages I-IV	0/5 healthy 0/4 CP	related to tumor size/persistent circulating mutant KRAS DNA 3 out of 15 patients after surgery and/or chemoradiation	Yamada et al. 1998 [58]
22/47 (47%)	allele-specific PCR followed by gel electrophoresis	stages I-IV	4/31 CP	patients with CP did not get pancreatic cancer in 36 months follow up time/no association with tumor stage or survival	Maire et al. 2002 [56]
20/56 (36%)	RT-PCR using PNA clamping	operable + unoperable disease	0/13 CP	no association with tumor resectability or survival	Dabritz et al. 2009 [111]
9/26 (35%)	mismatch ligation assay	stages I-IV	-	persistent circulating mutant KRAS DNA after surgery in 4 out of 9 patients/no association with tumor stage, grade or metastasis	Uemura et al. 2004 [112]
30/91 (33%)	PCR followed by sequencing	unresectable disease	-	related to stage, metastasis and survival	Chen et al. 2010 [59]
12/44 (27%)	PCR-RFLP followed by gel electrophoresis	stages I-IV	0/4 healthy 2/37 CP	no association with tumor size, however related to stage, metastasis and survival	Castells et al. 1999 [57]

(RT) PCR = (real time) polymerase chain reaction

CP = chronic pancreatitis

RFLP = restriction fragment length polymorphism

MASA = microsatellite associated sequence amplification

PNA = peptide nucleic acid

Summary of published reports on circulating miRs in patients with pancreatic cancer and benign pancreatic pathology.

Pancreatic cancer vs. benign pancreas pathology (n)	Circulating miRs	Pancreatic cancer vs. healthy pancreas (n)	Circulating miRs	Remarks	Reference
-	-	28 vs. 19	miR-21, miR-210, miR-155, miR-196a (all ↑)	-	Wang et al. 2009 [113]
-	-	28 vs. 19	miR-200a, miR-200b (both ↑)	benign pathology vs. healthy miR-200a, miR-200b (both ↑)	Li et al. 2010 [17]
-	-	50 vs. 10	miR-21, (↑)miRlet-7d, miR-146a (both ↓)	prognostic, survival	Ali et al. 2010 [101]
-	-	22 vs. 25	miR-210 (↑)	no correlation with CA19-9 levels	Ho et al. 2010, [see below in references]
6 vs. 8	miR-100a, miR-10 (both ↑)	-	-	miR-10 (↑)homology with pancreatic cancer-bearing mice/miR-155 (↓) tumor specific genecitabine response in pancreatic cancer-bearing mice	LaConti et al. 2011 [107]
-	-	36 vs. 30	miR-18 (↑)	miR-18 (↑) after surgery	Morimura et al. 2011 [104]
140 vs. 111	miR-21, miR-155, miR-16, miR-181a, miR-181b, miR-196a (all ↑)	140 vs. 68	miR-21, miR-155, miR-16, miR-181a, miR-181b, miR-196a (all ↑)	benign pathology vs. healthy: miR-155, miR-181a, miR-181b, miR-196A (all ↑)	Liu et al. 2012 [93]
41 vs. 35	miR-24, miR-134, miR-146a, miR-378, miR-484, miR-628-3p, miR-1290, miR-1825 (all ↑)	41 vs. 19	miR-24, miR-134, miR-146a, miR-378, miR-484, miR-628-3p, miR-1290, miR-1825 (all ↑)	miR-1290 and miR-486-3p prognostic	Li et al. 2013 [105]
109 vs. 38	miR-182 (↑)	109 vs. 50	miR-182 (↑)	prognostic	Chen et al. 2014 [115]
77 vs. 67	miR-10b, miR-30c, miR-106b, miR-132, miR-155, miR-181a, miR-181b, miR-196a, miR-212 (all ↑)	77 vs. 71	miR-10b, miR-30c, miR-106b, miR-132, miR-155, miR-181a, miR-181b, miR-196a, miR-212 (all ↑)	benign pathology vs. healthy miR-212, miR-155 (both ↑)	Cote et al. 2014 [92]
-	-	11 vs. 11	miR-642b, miR-885-5p, miR-22 (all ↑)	-	Ganepola et al. 2014 [90]
-	-	49 vs. 27	miR-492, miR-663a (both ↓)	-	Lin et al. 2014 [116]
32 vs. 12	miR-483-3p (↑)	32 vs. 30	miR-21, miR-483-3p (both ↑)	miR-21 prognostic	Abue et al. 2015 [102]
198 vs. 21	miR-6826-5p, miR-6757-5p, miR-3131, miR-1343-3p (all ↑)	100 vs. 150	miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, miR-4634, miR-7114-5p, miR-4476 (all ↓)	pancreato-biliary cancer taken together	Kojima et al. 2015 [91]