Introduction

Adapted from:

Circulating biomarkers to monitor cancer progression and treatment.

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Circulating DNA and Micro-RNA in Patients with Pancreatic Cancer.

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INTRODUCTION

Tumor Heterogeneity

Cancer presents with dynamic intratumoral and interlesional heterogeneity [1,2]. This process is driven by the genomic instability and rapid proliferation of cancer cells. Over time, cancer cell populations become even more diverse, known as temporal heterogeneity [3]. Tumor heterogeneity follows Darwinian evolutionary principles, where cancer cell subpopulations with optimal phenotypes including distinct surface marker expression, metabolism, proliferation, apoptosis, invasion, angiogenesis, drug sensitivity, and antigen presentation survive in a given tumor [4,5]. Tumor heterogeneity that enables malignant progression and metastatic spread by evolutionary selection is also the major cause of emergent resistance during cancer treatment. Yet, we rely on few standard diagnostic tumor biopsies for the characterization of a given cancer. These biopsies will provide only a partial characterization of the overall makeup of the dynamic systemic disease.

Circulating biomarkers

Tissue biopsies are invasive, expensive and represent a single snapshot in time of an evolving disease. The capturing and analysis of blood-based biomarkers is an alternative method to gain insight into the molecular makeup of cancer in a given patient. The major strength of circulating biomarkers is the possibility to compare serial samples from the same patient and thus generate a molecular readout of disease progression and therapy resistance in real time. Technologies have been rapidly developed over the past decade enabling the assessment of cell free nucleic acids. Extracellular nucleic acids, such as DNA and microRNAs (miRs), are found in almost all human bodily fluids and the circulation provides an attractive alternative to tumor tissue biopsies (Figure 1). The term "circulating" refers to the portion of extracellular molecules that are transported in the cell-free blood compartment. Identification of patient specific cancer markers is crucial for the improvement of controlling this disease. It is conceivable that blood samples can reflect the molecular profile of a primary tumor as well as residual cancer cells that are not accessible physically or below detection on radio graphical imaging. Liquid biopsies can be taken repeatedly to follow evolution of the disease. Circulating DNA and miRs are relatively stable in plasma and serum samples, and easy to quantitate by PCR or sequencing methods. Still, the interpretation of circulating nucleic acids remains challenging. Extensive validation and protocol standardization is required before circulating nucleic acid analysis can be used as clinically approved markers. Circulating miRs represent the tumor and the host response to malignant lesions. In contrast, circulating tumor-specific DNA harbors the molecular characteristics from the cancer cells such as mutations.

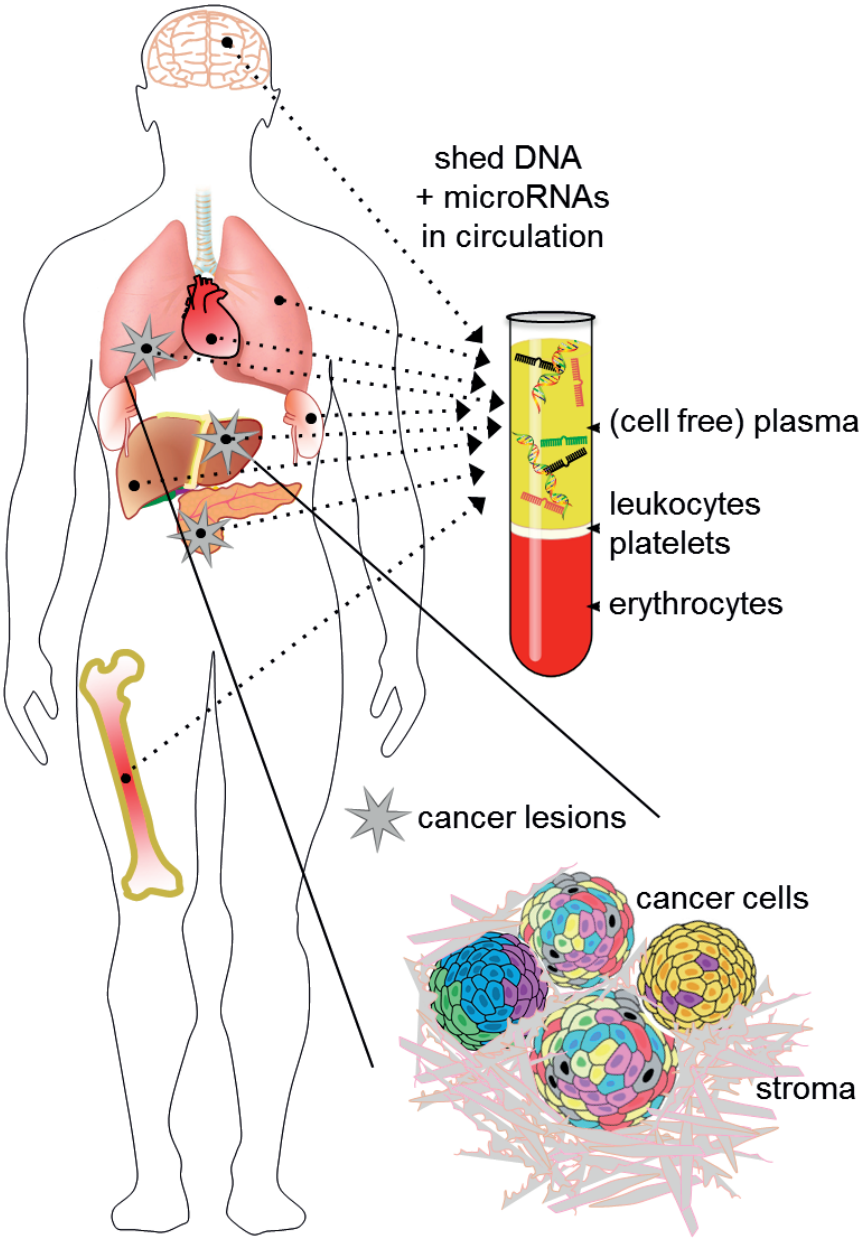

Figure 1. Release of cell free DNA and miR into the circulation. Healthy organs e.g. brain, lung, heart, kidney, liver, bone marrow shed wild type DNA and microRNAs into the bloodstream as indicated by the dotted arrows. The cancer lesions contain cancer cells (colored) and stromal cells (grey). Tumor-derived mutated DNA originates from the heterogeneous cancer cell populations and is shed into the circulation as cell-free (cf) DNA. MicroRNAs derived from blood cells, normal and tumor tissues reflect the composite of signaling in cancer cells, stroma and normal tissues. Double helices in the plasma compartment indicate cfDNA, short, single-stranded nucleic acids in different colors indicate miRNAs.

Circulating DNA

Cell-free DNA

All human cells can release DNA into their extracellular environment. Extracellular DNA has been detected in saliva, mucus, pancreatic juice, lymph, breast milk, cerebrospinal fluid, urine, amniotic fluid, and blood. Necrosing or apoptosing cells are thought to be the major source of cell-free DNA, however active DNA release via extracellular vesicles or protein complexes has been observed in cells such as lymphocytes and cancer cells[6,7]. DNA in the cell-free compartment of the blood, i.e., the plasma or serum, is called ccfDNA. In order to study ccfDNA, blood samples collected with standard collection tubes must be rapidly processed, to allow for reproducible assessments of ccfDNA levels. If plasma or serum are not separated from the cells within short order (~2 hours), dying cells can shed their DNA as well as DNA degrading enzymes and thus bias the levels of ccfDNA. Some collection tubes that capture and fixate the blood components appear to mitigate this problem and allow for shipping and later (within 72 hours) separation by centrifugation [8]. CcfDNA can be encapsulated in lipid membrane microvesicles, trapped by leukocytes, bound to nucleosomes, serum- and/or lipoproteins. Studies on the half-life of ccfDNA have described times ranging from minutes to 13 hours. This discrepancy is not surprising, given the multitude of possible ccfDNA carriers and factors involved in ccfDNA clearance. When ccfDNA is bound to protein complexes, or inside membrane vesicles, the DNA is less vulnerable to degradation. Phagocytes clean up dead cells and nucleases in the blood and rapidly degrade ccfDNA, whereas the liver and kidneys metabolize it. These events occur at different rates, depending on (patho)physiological conditions of the individual at that time. As the knowledge on ccfDNA has dramatically increased over the past decade, it became clear that levels of DNA in the blood are a comprehensive indication of the present physiological and pathological processes throughout the body. CcfDNA can impact the immune system and signals through Toll-like receptors on dendritic cells and macrophages. Both ccfDNA from the host and from microbial invaders can elicit inflammatory responses [9-11]. In the case of the autoimmune disease systemic lupus erythematosus (SLE), DNA-specific antibodies bind to the abundant extracellular, double stranded ccfDNA and form immune complexes that elicit inflammation. Moreover, patients with DNAse deficiency have increased susceptibility to autoimmune diseases such as SLE [12], due to the increased levels of ccfDNA. In the blood of healthy individuals, the vast majority of ccfDNA originates from erythrocytes, leukocytes, and endothelial cells [13,14]. In addition, ccfDNA is shed from cells of organs that incurred damage by hypoxia, trauma, inflammation, etc. [15,16]. CcfDNA levels are elevated after physical exercise [17-19], surgery [20], myocardial infarction [21], and many other disorders. Especially in patients with cancer, ccfDNA levels rise dramatically [22-24]. Ever since this observation was made in the late 1970s, ccfDNA has become a major player in the field of cancer biomarkers.

Circulating DNA levels in patients with cancer

The levels of ccfDNA in patients with cancer can be increased due to higher rates of cellular turnover and cell death in tumor tissues. Tumor-derived ccfDNA is termed circulating cell-free tumor DNA, in short 'ctDNA'. Cancer cells are not the only cells in tumors that contribute to the high levels of ctDNA: activated tumor-associated fibroblasts, endothelial cells, and immune cells also shed DNA. It is well established that ctDNA does not originate from intact circulating tumor cells: cell-free ctDNA levels are 100-1000 times more abundant than the intracellular DNA content of circulating tumor cells and the typical number of circulating tumor cells per mL of blood (<10), cannot account for the numerous micrograms of ctDNA per mL of blood observed in cancer patients [25,26]. CtDNA is smaller in size than ccfDNA from healthy individuals [27,28]. The sizes of ctDNA fragments range from 120-180 base pairs (bp) and peak below 150 bp [27,29,30]. The packaging of extracellular DNA is different for cancer cells from different origin and also depends on the extent of phagocytosis by immune cells. A small portion of ctDNA is packaged into exosomes and can measure 100-2500 bp; even larger DNA strands can bind to the outer membrane of exosomes [31]. Aside from the release mechanisms and packaging of ctDNA, the quantity of ctDNA is dependent on the type, location, vascularity, and size of the tumor. For instance, hypoxia is a major driver of ctDNA release [32]. Thus, tumor load and ctDNA levels do not necessary correlate with each other. Instead of quantitating the levels of ctDNA, many studies focus on cancer cell-specific, qualitative characteristics of ccfDNA in cancer patients. Because ctDNA refers to the cell-free DNA portion in blood that is derived from the tumor it harbors the hallmark genomic alterations of the cancer cells from which the DNA is shed.

Circulating Mutant Tumor DNA

Mutations in ctDNA are often studied as qualitative and quantitative biomarkers in cancer patients. Advances in PCR-based detection assays have allowed for relatively easy mutant ctDNA measurements. When quantitating known mutations, methods such as droplet digital PCR allow for mutation detection as low as 1 per 10.000 copies of wildtype DNA. Similar detection thresholds are met when screening for a broader range of genomic alterations, using targeted panels of amplicon deep sequencing. The number of mutations vary by up to 1000-fold among specimens from a given cancer type [33]. Although mutant ctDNA originates from cancer cells, tumors consist of heterogeneous cancer subpopulations. This intratumoral heterogeneity implies that different cancer cells harbor different sets of mutations. Therefore it is prudent to monitor more than one gene mutation when attempting to correlate clinical outcomes to mutant ctDNA levels. Moreover, the stroma component of cancers is important to consider when quantitating mutant ctDNA. The highly reactive stroma in pancreatic cancers for instance, could lead to a shift in the mutant ctDNA fraction compared to wildtype ctDNA. In contrast to studies in pancreatic cancer, which have shown that fewer myofibroblasts in tumors are associated with poor survival [34], in colorectal

cancer it has been shown that a lower cancer cell-to-stroma ratio is associated with worse outcomes [35]. Likewise, high levels of cancer-associated fibroblasts in esophageal cancer are correlated with a worse clinical outcome [36].The levels of mutant ctDNA in the background of wildtype ctDNA could be misleading when correlations are made between clinical outcomes and mutant fractions of ctDNA [16]. In early stages of cancer, mutant ctDNA may be below the detection limit due to a low tumor load. Also, missed ctDNA can be caused by clonal heterogeneity of a tumor, or because a subpopulation carrying the mutation has a low apoptosis rate. In some cases the total ctDNA level is the superior marker of cancer load [37], though a particular mutant ctDNA can be informative if an abundant gene mutation is assessed [22]. As discussed above, biological factors such as tumor vascularity, phagocytosis of dead cells, ctDNA clearance by the kidneys and liver, as well as the sensitivity of the detection assay will affect the measurements of mutant DNA. Even copy numbers of the mutant allele can be different in cancer cells from the same tumor. In colorectal cancer patients with detectable mutant KRAS and BRAF ctDNA, mutant allele fractions in plasma can range from 0.13% to 68.77%, as determined by Intplex PCR [38]. Therefore, most studies separate patients based on the presence or absence of mutant ctDNA for the cancer related genes monitored rather than the abundance of the mutant allele fraction.

Circulating microRNAs

MiR biogenesis

Mature microRNAs (miRs) are highly conserved short strands of non-coding RNA, derived from hairpin precursor transcripts [39]. After cleavage of primary microRNA (pri-miRNA) transcripts by the Drosha/DCGR8 complex, nuclear-to-cytoplasmic transport, and maturation with DICER1[40,41], 21–24 nucleotide long, double stranded mature miRs are formed. One of the mature miR strands binds predominantly to the 3' untranslated region (UTR) region of mRNA to regulate protein translation. Additionally, miRs can also bind to the open reading frame (ORF) or 5'UTR of target mRNAs to repress or activate translational efficiency [42-44]. The discovery of small RNAs that are involved in translation regulation via an antisense RNA-RNA interaction was first described in Caenorhabditis elegans [45]. To date, more than 2600 human mature miRNAs have been identified and annotated [46], with more than half of human protein-coding genes likely regulated by a miR [47].

MiR expression alterations in cancer

MiRs are dysregulated in cancer and play crucial roles in cell proliferation, apoptosis, metastasis, angiogenesis and tumor-stroma interactions [48]. Dysregulated miRs can function both as oncogenes (e.g. miR-155; miR-21, miR-221; miR-222, miR-106b-93-25 cluster; the miR-17-92 cluster) and tumor suppressors (e.g. miR-15; miR-16; let-7; miR-34; miR-29; miR-122, miR-125a-5p and miR-1343-3p), depending on their downstream targets [49,50]. Many human miR genes are located on chromosomal sites that are susceptible to

chromosome breakage, amplification and fusion with other chromosomes [51]. Additionally, alterations in RNA binding proteins and cell signaling pathways contribute to cancer through miRNA expression changes as well as mutations in core components of the miR biogenesis machinery that can promote oncogenesis [40]. It has recently been shown that mutant KRAS in colon cancer cell lines leads to decreased Ago2 secretion in exosomes and Ago2 knockdown resulted in decreased secretion of let-7a and miR-100 in exosomes whilst cellular levels of the respective miRs remained unchanged compared to control cells [52]. A systematic expression analysis of 217 mammalian miRs from 334 samples, including multiple human cancers revealed extensive diversity in miR expression across cancers, and a large amount of diagnostic information encoded in a relatively small number of miRs. More than half of the miR (129 out of 217) had lower expression levels in tumors compared to normal tissues, irrespective of cell types [53]. miR expression profiles allows classification of poorly differentiated cancers and identify tumors of unknown tissue origin [53]. In subsequent studies, profiling miR expression improved cancer diagnosis and helped identify the tissue of origin in carcinoma with unknown primary site by standard histology or immunohistological analyses [54,55].

Cell-free miRs

MiRs are present and stable in the peripheral circulation. The first report on miR expression in the circulation in 2008 described detection of four placenta-associated miRs (miR-141, miR-149, miR-299-5p, and miR-135b) in maternal plasma during pregnancy, after which the level decreased following delivery [56]. In 2008, a study demonstrated increased levels of circulating miR-21, miR-155 and miR-210 expression in patients with diffuse large B-cell lymphoma (DLBCL) compared to healthy controls [57]. Mitchell et al. also showed that circulating serum miR-141 could distinguish patients with advanced prostate cancer from healthy controls [58]. The vast majority of research on circulating miR signatures in oncology is focused on diagnostics [59], in which patients with cancer are compared to healthy individuals. Given the profuse inter-individual differences in genetic background of individual patients in addition to the heterogeneous nature of cancer, using cf-miR as cancer diagnostic biomarkers will remain challenging. 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 [60]. The origin of cf-miR is heterogeneous. miR-21 is a good example to illustrate this point. Although the release of miR-21 into the circulation is correlated with a multitude of cancer types, it is also highly expressed in activated T-cells and associated with inflammation and wound healing [61,62]. Elevated circulating miR-21 levels do not merely reflect tumor presence. They can also reflect the host response to the tumor, which is important in predicting disease progression. Moreover, there are often discordances between cf-miRNA signatures and the paired tumor tissue [59]. Assuming that the quality of miRNA measurements is not determined by the

efficacy of RNA extraction, this suggests that cancer-associated cf-miRNA deregulations is more likely to reflect the systemic response to the presence of cancer. Indeed, several studies have shown that cf-miRNAs are predominantly derived from blood cells [63] and the endothelium [64] in addition to the tumor.

Cell-free miRs as prognostic biomarkers

Cancer progression and systemic drug therapy involve many organ systems and are not limited to the primary tumor. This makes cf-miRNA attractive biomarkers for cancer progression and drug efficacy monitoring. For instance, in serum obtained pre-surgically from patients with early stage colorectal cancers, a panel of 6 circulating miRNAs can predict cancer recurrence [65]. In pancreatic cancer patients, an expression signature of 6 plasma miRs was associated with worse overall survival [66]. Changes in cf-miRNA patterns within the same patients can be monitored over time during therapy. The growing evidence of the utility of cf-miRNA as cancer therapy response indicators has been accumulating during the last few years [67-69]. Cf-miRNAs are likely to surpass the clinical utility of conventional protein markers such as CA-125, CA19-9, PSA and radiographical imaging, which have low sensitivity and specificity for detection of minimal residual disease and are not designed to characterize cancer at the host response level.

OUTLINE OF THIS THESIS

Cancer is a heterogeneous disease which is constantly evolving. Cancer cell subpopulations have distinct genomic and phenotypic properties and respond differently to treatment. **Chapter 2** assesses clonal subpopulations of pancreatic cancer from a transgenic mouse that develops pancreatic cancer induced by mutations in *Kras* and *Trp53*. We characterized the clonal cells on the genomic, phenotypic and drug-sensitivity level. More importantly, we studied the sensitivity of the clonal cells to different types of anti-cancer drugs in the context of the mixed heterogeneous tumors in the presence of an intact immune system.

In contrast to tumor biopsies, 'liquid biopsies' are new in the field of biomarkers and can be obtained repeatedly by blood draws to follow cancer evolution. In order to follow the dynamics of cancer over time we studied the mutation patterns in circulating tumor DNA over the course of cancer progression in patients with colon and pancreatic cancer in **Chapter 3.** Plasma DNA mutations in 56 cancer-associated genes before surgical removal were compared to the mutations found in the primary tumors. Moreover, at time of metastatic disease, the plasma DNA mutations were compared to those at time of the primary disease.

MiRs control genes in cellular processes such as apoptosis, proliferation and inflammation and miRs are dysregulated in in diseases such as cancer. In contrast to circulating

tumor DNA, which can indicate presence of cancer cells, circulating miRs could provide information about the host's response to cancer. In **Chapter 4** we analyzed the changes in serum miRs in patients with pancreatic cancer after resection, and compared the serum miR expression of patients with short versus long progression free survival. The results were compared to circulating miRs that are altered in KPC transgenic mice that developed metastatic pancreatic cancer. We further assessed the expression of two miRs that are associated to cancer progression in the patients' tumor tissues by in situ hybridization.

In **Chapter 5** we studied circulating miRs in patients with breast cancer undergoing kinase inhibitor treatment. Dovitinib is a multi-kinase inhibitor impacting angiogenesis and the tumor-stroma crosstalk. We measured serum miR expression over the course of dovitinib therapy to assess whether circulating miRs could indicate drug response or resistance.

The work presented in this thesis is summarized and discussed in **Chapter 6.**

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