Summary and Discussion

SUMMARY AND DISCUSSION

As early as 1976 scientists noted that genomic instability of a proliferating cancer population is followed by additional genetic diversity under the selection pressures in the tumor environment, resulting in multiple heterogeneous subpopulations [1]. Cancer heterogeneity is the main cause of drug therapy resistance. Therefore, we conducted a study that could provide insights for the improvement of effective cancer therapies, described in **chapter 2**. We generated a model of cancer heterogeneity by isolating and characterizing clonal cancer cell lines from a transgenic *LSL-Kras*$^{G12D/+;}$ *LSL-Trp53*$^{R172H/+;}$ *P48*-Cre (KPC) mouse pancreatic cancer (PDAC). We found that the clonal cell lines from the same tumor harbor common ancestor mutations and more importantly, each have a set of unique signature mutations. Not only do these clonal cell lines grow at different rates in vitro, their sensitivity to anti-cancer drugs of various mechanisms of action was distinct.

The novelty of our study lies in the fact that we can mix the clones back together and study their drug sensitivity in the context of a heterogeneous population where paracrine crosstalk is possible. The unique clonal mutations are used for the measurement of clone abundance in the mixed tumors by next generation sequencing (NGS). Although each clone is driven by the RAS-RAF-MEK-ERK signaling pathway due to the *Kras* G12D mutation, the clones are differentially sensitive to the MEK inhibitor trametinib on their own. What was even more striking is that the clonal sensitivity to trametinib in vitro was altered when clones were mixed back together and thus subjected to paracrine signaling. This finding was confirmed when using the conditioned media from the clone mixture, and shows that pharmacological screenings in vitro using cell lines such as the NCI-60 panel should rather be performed in heterogeneous mixtures of cell lines to improve the predictive validity.

We studied the clone mixture as allograft tumors in immune competent syngeneic mice, and noticed a difference in growth rate amongst the clones. Moreover, gemcitabine treatment had a stimulating effect on the slow growing clones C5 and D10 that were underrepresented in control tumors. Gemcitabine treatment allowed these clones to gain a 4-5 fold increase in growth as compared to the control conditions. Others reported similar findings in minor dormant human colorectal cancer clones that can become dominant and reinitiate tumor growth after chemotherapy [2]. Strikingly, two other clones that were sensitive to gemcitabine in vitro, were not responding to gemcitabine in the mixed tumors in presence of tumor stroma. This suggests that the crosstalk with the tumor stroma offers paracrine factors that protect these clones from the chemotherapy.

A comparison of clonal effects of MEK inhibitor in the mixed culture in vitro and in the tumors showed a discordant result for clone G9 that moved from sensitive in vitro to resistant

in the tumors and clone C8 that moved in the opposite direction. Others have also described that the tumor environment alters the drug sensitivity of cancer cells [3,4]. Yet, until now, most cancer drug screens are performed in vitro, or in xenograft tumors with homogeneous cancer cell lines in immune compromised animals.

The beauty of our clonal allograft model lies in the fact that it allows for analyses of heterogeneous tumors in the context of an intact immune system. The striking differences in α-PD-1 efficacy towards clones present in the heterogeneous tumor mix which we observed in our study provides some interesting insights that may allow to overcome resistance to checkpoint blockade. One of the clones, C8 is highly sensitive to α-PD-1 treatment and on its own attracts the highest number of CD8+ PD-1+ T-lymphocytes towards the tumors. We conducted a co-culture experiment in vitro with primed mouse T-lymphocytes and the clonal PDAC cell lines to corroborate this finding. In order to generate enough vital T-cells we used the caecal patches of tumor bearing mice as source of tumor-reactive T-cells, instead of the commonly used tumor infiltrating lymphocytes which are often exhausted [5]. We observed that the pancreatic cancer clones elicit different levels of T-cell activation, measured by Interferon-γ secretion. Strikingly, growth of one clone, D10 was stimulated in vivo by anti-PD-1 treatment and we are currently exploring this further. Moreover, we found that that the caecal patch, the equivalent of the human vermiform appendix [6,7], contains pancreatic cancer reactive T-cells. We are the first to have shown this and we are currently exploring the reactivity of T-cells from the vermiform appendix of patients with locally advanced pancreatic cancer.

To conclude chapter 2, we found that the composition of heterogeneous cancers is affected by crosstalk amongst the cancer subpopulations as well as the host environment that includes the immune system as a major player. We developed an in vivo model that allows for the quantitation of clonal cancer subpopulations in heterogeneous tumors, growing in immune competent. Our model is suited for the assessment of stromal and immune modulators and their impact on growth of heterogeneous cancer cells.

Cancer is a heterogeneous and dynamic disease. Yet, until today, molecular analyses of cancer are still performed using small tissue biopsies. In **chapter 3** we sought to assess the changes in the mutational makeup of colon and pancreatic adenocarcinoma between the time of primary tumor surgery and detection of metastatic disease using circulating cell free DNA. In this study we also provide a direct comparison of mutation detection in primary tumor DNA and plasma DNA at diagnosis. For this, amplicons covering 263 mutations in 56 cancer-associated genes were analyzed by next generation sequencing. We found that on average less than half of colon tumor mutations were detected in the plasma at time of diagnosis. For pancreatic cancer this percentage was even lower: less than 30% of pancreatic

tumor mutations were detected in the circulating DNA. Whether ctDNA can fully replace or complement tissue analyses in all cancer types remains controversial, especially in early stage diseases.

Disadvantages of circulating tumor DNA (ctDNA) include the fact that high background levels of wildtype DNA can lead to missed ctDNA detection. Also, the genetic make-up of dying cells is dominant in ctDNA, and that of vital tumor cells may be underrepresented. Others have shown that in formalin fixed-paraffin-embedded cancer tissues and plasma from patients with different types of cancer an overall concordance of 60% in mutations of 19 genes analyzed was found [8]. Another prospective study with matched plasma and tissue samples of 75 cancer patients with different tumor types (61 metastatic and 14 clinical stage II patients) looked at 54 genes with digital deep sequencing technology. In the tumor tissues, at least one somatic mutation in 44 of 61 samples (72.1%) was found. From those 44 tissue samples with mutations, ~66 % of the matched patients had detectable mutations in the plasma DNA [9]. The sensitivity of ctDNA mutation detection improves as the tumor load increases. An extensive study including different cancer types showed that the fraction of patients with mutant ctDNA increases with cancer stages: 47% of patients with stage I cancers of any type had detectable ctDNA, whereas the fraction of patients with detectable ctDNA was 55%, 69%, and 82% for patients with stage II, III, and IV cancers, respectively [10].

On the other hand, we detected 71-78% of mutations in the plasma DNA of patients at time of diagnosis that were not detected in the tumor tissue. One reason for the discordance is the small size of the tumor biopsy. The tumor biopsies are merely a tiny fraction of the entire heterogeneous tumor. Moreover, the patients may already have micrometastases that are not detected on the CT scans, but already release mutant DNA in the circulation. Overall the presence of ctDNA in patients with progressive cancer provides a median lead-time of detection of 8-9 months over computed tomography (CT) scans [11,12]. A recent study in patients with non-small cell lung cancer patients revealed that 18 patients had detectable *EGFR* T790M ctDNA in their plasma, while the tumor DNA was negative for *EGFR* T790M genotyping [13]. This demonstrates that tissue biopsies may not represent the entire cancer cell population, or result in false negative mutation results due to low abundance of cancer cells in the specimen. One of the caveats is that circulating DNA mutations may not originate from cancer cells. It is worth noting that somatic mutations found in skin biopsies from healthy individuals can approach the mutation levels seen in cancers [14], however, mutant DNA from healthy tissues is typically below detection in the circulation. This is likely due to the induction of senescence in normal cells with mutant DNA rather than cell death and shedding of their DNA, and the low frequency of mutations at a variety of random sites in the genome. Cancer cells on the other hand are highly abundant, and often

have non-synonymous mutations. More importantly, cancer cells have a high turnover rate and thus a high death rate.

We assessed the change in mutations from the time of primary resectable cancer diagnosis to the recurrence of cancer after surgical tumor removal and adjuvant therapy in patients with colon and pancreatic cancer. This analysis reflects the evolution of primary to recurrent cancer. After metastasis, new ctDNA mutations are gained both in colon (33.8%) and pancreatic cancer (62.6%) and were not detected at the time of diagnosis of the primary cancer. Complementary to the gain of mutations after metastasis, we also observed a loss of approximately half of the ctDNA mutations. The analysis of a relatively broad panel of cancer-related genes is feasible for ctDNA and would allow monitoring of changes in the molecular makeup over time and under therapy.

Summarizing chapter 3, we found that circulating tumor DNA appears to represent the heterogeneity of colon and pancreatic cancer more extensively than tumor tissue DNA. By comparing the evolution of mutant ctDNA over the course of treatment, emergence of mutations that are associated with therapy-resistant cancer can be studied easily by collecting repeated patient blood samples. It is highly likely that liquid biopsies will become essential in cancer patient diagnostics and follow-up in the very near future.

Until now, the clinical follow up of patients with pancreatic cancer after surgery is not standardized in the Netherlands and consists mainly of clinical physical examination and at times radiographical imaging by CT-scans [15]. Simple repeated blood draws followed by biomarker analyses could vastly improve patient monitoring and prediction of outcome. Circulating microRNAs (miRs) are suitable candidates due to their high stability in the circulation and their importance in pathophysiology. In **chapter 4** we measured serum miRs in patients with treatment-naïve resectable PDAC before and after surgical tumor removal and compared the changes of miR levels to the progression free survival of the patients. Importantly, we performed a cross-species comparison of serum miR expression: we analyzed the serum miR levels in KPC mice that develop metastatic pancreatic cancer. The prognostic miRs we identified in the patients were also correlated to PDAC metastases in the KPC mice, confirming their importance in pancreatic cancer progression. From the 250 miRs we profiled, the expression levels of only miR-125b-5p and miR-99a-5p could significantly separate patients based on progression free survival after surgery.

Next, we assessed whether miR-125b and miR-99a-5p are expressed in the resected pancreatic cancer tissues by using in situ hybridization. To our surprise, the cells that express the miRs are very low abundant, and are only present in the pancreatic stroma. It is becoming increasingly clear that circulating miRs represent a rich source of information about the

status of the immune system. MiRs in the serum originate mainly from endothelium and blood cells [17,18]. The circulating miRs are transferred from cell to cell and can elicit immune modulation [19,20]. Serum miRs interact more with immune-related mRNA genes that with non-immune related genes [21]. We found that the high miR-125b-5p and miR-99a-5p expressing cells are limited to inflammatory infiltrates in the connective tissue of the pancreatic tumors. After staining the consecutive tumor sections for CD79A we found that the high miR-125b-5p and miR-99a-5p expressing cells are in very close proximity to CD79A positive cells which are part of the B-lymphocyte lineage. This underlines the importance of circulating microRNAs in the host response to cancer. Our future aim is to conduct a histopathological validation to study the co-localization of miR-125b-5p and miR-99a-5p in various subtypes of immune cells to gain understanding in the immune cell function alterations in patients with cancer.

Although there are a multitude of studies comparing circulating miR levels in pancreatic cancer patients to healthy controls [22,23], there has been very little to no research that compares the levels of miRs after surgical removal of primary pancreatic cancers. In our study we compared serum miRs pre and post-surgery from treatment-naïve patients that were operated at the Erasmus Medical Center between 2013 and 2017. Recently it became clear that patients with (borderline) resectable PDAC that undergo preoperative chemo/radiotherapy have a better survival [24,25]. From now on all patients with (borderline) resectable PDAC in the Netherlands will be offered to receive preoperative chemotherapy, if the performance status of the patient permits the therapy, and the patient is willing to undergo systemic treatment. Chemotherapy has a vast impact on the immune landscape [26,27]. Whether serum miR-125b-5p and miR-99a-5p are also predictive of disease progression after surgery in pre-treated patients remains to be evaluated. Therefore we are currently collecting blood samples to evaluate the changes in the serum miRs of patients with (borderline) resectable patients who undergo pre-operative FOLFIRINOX chemotherapy or gemcitabine/radiotherapy.

In **Chapter 5** we analyzed plasma miRs in patients with hormone receptor positive, metastatic breast cancer with prior disease progression during aromatase inhibitor therapy in a phase I/II trial with the multiple tyrosine kinase inhibitor dovitinib. The dual inhibition of FGF and VEGF signaling by dovitinib enhances the antitumor effects through the targeting of immune evasion and angiogenesis in the tumor microenvironment [28]. We hypothesized that expression of circulating miRs can provide an accurate read-out of these effects of dovitinib. Changes in plasma miR levels were measured by quantitative RT-qPCR before and after treatment with dovitinib and compared to changes in tumor sizes. The altered expression patterns observed for the six circulating miRs (miR-21-5p, miR-100-5p, miR-125b-5p, miR-126-3p, miR-375 and miR-424-5p) separated patients with resistant disease

from those with drug responsive disease. What was striking is that in a patient with long term response to dovitinib we observed a change in the circulating miR signature before the CT scan showed a reduced effect of the treatment at 24 weeks. Although this study consisted of a low number of patients, we conclude that changes in the expression patterns of circulating miRs can be indicators of dovitinib response, and this research merits the understanding of the host response to metastatic cancer.

CONCLUSIONS

The research in this thesis provides rationale for the implementation of circulating nucleic acid analyses into the standard of care of patients with cancer. Until now, cancer tissue specimens are used for molecular analyses of cancer, and clinical follow-up consists mainly of radiographical imaging. Cancer is a heterogeneous, dynamic disease that constantly changes over time and across different locations in the body. To improve our understanding in the molecular features of this disease as well as the response to cancer therapy, blood samples need to be taken repeatedly and analyzed for changes in circulating nucleic acids. Changes in circulating DNA mutations indicate clonal evolution of cancer populations and altered abundance of mutant circulating DNA suggests a change in disease burden. Moreover, changes in circulating DNA mutations reflect the emergence of resistant clones and prompt changes in treatment. In contrast to mutant DNA, circulating microRNAs take part in the extracellular crosstalk between cells and provide a readout of the organism's physiologic or disease state. Furthermore, changes in circulating miR patterns can reflect the immune landscape, treatment efficacy or resistance as well as adverse effects associated with the respective intervention. Thus, the combined serial analysis of mutant DNA and microRNAs in the circulation has the potential to provide a molecular footprint of cancer and can be used to monitor cancer progression as well as treatment responses in real time with a minimally invasive procedure.

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