DISCUSSION
Chapter 13

Discussion

Amir Avan\textsuperscript{1}, Thomas Würdinger\textsuperscript{2}, Gerrit Jan Schuurhuis\textsuperscript{3}, Godefridus J. Peters\textsuperscript{1}, Elisa Giovannetti\textsuperscript{1}

\textsuperscript{1}. Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands.
\textsuperscript{2}. Neuro-oncology Research Group, Departments of Neurosurgery and Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, The Netherlands.
\textsuperscript{3}. Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands.

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Discussion
An incidence rate nearly equal to its mortality rate demonstrates the aggressiveness and lethal nature of pancreatic ductal adenocarcinoma (PDAC). Most patients present with advanced disease (i.e., locally-advanced or metastatic) at diagnosis, and survival rate has not improved in the last decade, with less than 5% of patients alive five years after diagnosis. On one hand, such dismal outcome can be explained by the lack of biomarkers for early screening/diagnosis, together with the aggressive biological behavior, characterized by early metastatic spread and resistance to currently available chemotherapy regimens. On the other hand, prognostic and predictive biomarkers of treatment response are also missing, and most agents in clinical trials are selected on the basis of their activity in preclinical models that do not recapitulate the molecular and histopathological hallmarks of PDAC, thus creating a selection bias when choosing drugs for testing in the patients.

The research described in the current thesis was mainly focused on: (1) the elucidation of molecular mechanisms underlying the aggressiveness and chemoresistance of PDAC, in order to identify prognostic and predictive markers of treatment response; (2) the study of the therapeutic potential of novel anticancer agents; (3) the establishment of innovative in vitro and in vivo models of PDAC, starting from primary tumor cells, to test new (targeted) treatment strategies (Figure 1).

Figure 1. Overview of the aims of the current thesis.
Scope 1: Identification of prognostic or predictive markers of treatment response

Adjuvant or palliative chemotherapies slightly improve/prolong survival in the resected or unresected PDAC patients. However, most patients do not achieve clinical response and have a very short survival expectancy. Identification of novel predictive or prognostic biomarkers of chemotherapy is essential for better clinical management. The aim of the first part of the present thesis was to unravel predictive/prognostic biomarkers of chemotherapy activity to select patients with the highest likelihood of responding, while minimizing useless and toxic treatments, as described in chapters 2-6 and chapter 11.

Recently, the most relevant therapeutic improvement in metastatic PDAC has been obtained from the combination of cytotoxic agents, such as 5-fluorouracil with leucovorin combined with irinotecan and oxaliplatin, in the FOLFIRINOX regimen [1]. Moreover, Reni and colleagues have reported the results of five studies [2-6], including a phase III trial, demonstrating superiority over single-agent gemcitabine, of the four-drug regimens cisplatin–epirubicin–5-fluorouracil–gemcitabine, cisplatin–docetaxel–capecitabine–gemcitabine (PDXG) and cisplatin–epirubicin–capecitabine–gemcitabine (PEXG). Two recent surveys mirroring the clinical practice in the first-line therapeutic management of advanced PDAC suggested that four-drug combinations might yield a better outcome when compared to other regimens [6,7]. However, combinations of several cytotoxic agents are associated with increased hematologic or extra-hematologic side effects. Therefore, analysis of accessible biomarkers, such as germ-line polymorphisms, could provide a useful tool in the selection of the most appropriate chemotherapeutic regimen to be used in a patient-adapted way.

1.1. XPD-Lys751Gln polymorphism as a prognostic factor in gemcitabine-cisplatin polychemotherapy regimens in PDAC

In our previous pharmacogenetic study in 122 advanced PDAC patients treated with gemcitabine–cisplatin-based polychemotherapy, the Xeroderma Pigmentosum group-D (XPD) polymorphism at codon-751 (XPD-Lys751Gln) emerged as the most significant independent predictor for death- and progression risk, among 11 candidate functional polymorphisms [24]. Consistent with these findings, we also showed a significant association of XPD-Gln751Gln with shorter progression-free survival (PFS, \( P=0.02 \)) and overall survival (OS, \( P=0.04 \)) in 93 advanced non-small cell lung cancer patients treated with second-line carboplatin plus pemetrexed [8]. However, a recent meta-analysis of 12 studies demonstrated inconclusive data about this polymorphism in NSCLC [9]. These discrepancies could suggest that pharmacogenetic associations are not always reproducible in small size studies, with different clinical settings, tumor types, stage and treatment. Larger multicenter studies are essential to investigate the role of emerging biomarkers before planning of prospective trials.

Therefore in the chapter 2 of this thesis, we further investigated the prognostic role of XPD-Lys751Gln in 337 patients (247 treated with gemcitabine–platinum regimens and 90 treated with gemcitabine) with locally advanced or metastatic PDAC. Moreover, to test our hypothesis that XPD-Gln751Gln genotype was associated with a more efficient DNA repair after cisplatin exposure, we determined DNA damage capacity in lymphocytes harboring the different XPD-Lys751Gln genotypes.

In this study, we identified XPD-Lys751Gln polymorphism as a significant independent
predictor for death and progression-risk in PDAC patients treated with four-drug polychemotherapy regimens. In particular, patients carrying the XPD-Lys751Lys or Lys751Gln genotypes had a significantly longer median OS (log-rank-P<0.01). Similarly, the median PFS of patients harboring the XPD-Gln751Gln genotype was significantly shorter than the median PFS of XPD-Lys751Lys or XPD-Lys751Gln patients. Moreover, The XPD-Gln751Gln genotype was markedly associated with increased risk of death (HR=2.1) as well as with increased risk of progression (HR=1.9) at multivariate analysis. In addition, the analysis of DNA damage in lymphocytes supported the association of XPD-Gln751Gln with greater resistance to cisplatin-induced damage. These results might be explained by the central role of XPD in DNA-repair and platinum activity, and may have important clinical applications. Indeed, the analysis of the polymorphism by a simple blood test offers an innovative tool for optimizing palliative chemotherapy in patients with advanced PDAC.

1.2. EZH2 is a prognostic factor for locally-advanced and metastatic PDAC

Several genetic alterations have been associated with the aggressive behavior and chemoresistance of PDAC [77], while epigenetic factors recently emerged for their roles in tumor progression. In this context, Enhancer of Zeste Homolog 2 (EZH2) is becoming increasingly acknowledged as a prognostic biomarker in radically resected PDAC patients [11]. Therefore in the chapter 3, we describe the prognostic value of EZH2 in the PDAC patients with locally advanced or metastatic disease. Moreover, since recent studies suggested a role for candidate polymorphisms of EZH2 in lung cancer risk and colorectal cancer prognosis [12,13], we investigated the correlation of candidate polymorphisms and EZH2 expression with outcome. EZH2 mRNA and protein levels were evaluated in two cohorts of 32 laser-microdissected specimens and 25 samples collected in a Tissue Microarray (TMA), while polymorphisms analyses were performed in 340 patients (247 treated with four-drug regimens, as reported in the previous chapter 2, and 93 treated with gemcitabine).

Patients were divided into two subgroups according to the median EZH2 mRNA expression and evaluated for clinical outcome after gemcitabine chemotherapy. The high EZH2 expression group had a significantly poorer prognosis. Immunohistochemistry showed a variable protein expression in the patient samples, related to the mRNA expression. Indeed, the tissues characterized by high EZH2 expression, showed a strong and diffuse staining, while the tissues with low EZH2 expression had only few scattered positive cells with a weak nuclear staining. EZH2 protein expression was also related to outcome, and similar results were observed in the TMA samples. EZH2 expression was lower in grade-I/II (N=13) than grade-III (N=19), while no difference was observed according to other clinicopathological parameters. In addition, the rs6950683 C/C genotype was associated with a markedly higher EZH2 expression, and patients harboring this genotype had a trend towards a significantly shorter OS. However, no significant differences were observed in OS for EZH2 polymorphisms in two larger cohorts of patients, treated with gemcitabine-alone and with polychemotherapeutic regimens from a multicentric series. In conclusion, EZH2 expression emerged as a prognostic factor for locally advanced or metastatic PDAC, but candidate polymorphisms could not predict the outcome. Other factors involved in the EZH2-oncogenic pathways and detectable in accessible samples sources, such as candidate miRNA (ie. miR-101) enriched tumor-derived exosomes in peripheral blood [14], should be investigated in order to improve the clinical management of advanced PDAC patients.
1.3. Do observational studies provide a strong rationale for future trials to validate the best markers for personalized treatment of PDAC patients?

Recently, Fisher and colleagues showed that high tumor expression of ribonucleotide reductase subunit-M2 (RRM2) and excision repair cross-complementing group-1 (ERCC1) correlated with reduced survival, as determined by immunohistochemistry [15]. In chapter 4, we discuss these findings in comparison with our previous results. In particular, in our previous studies on mRNA expression of 7 genes involved in gemcitabine activity in laser-microdissected PDACs, we did not observe differences in RRM2 expression using a specific quantitative-RT-PCR technique, while patients with higher levels of human equilibrative nucleoside transporter-1 (hENT1) had significantly longer OS [16]. In agreement with these data, other studies showed that patients with high hENT1 expression benefit from gemcitabine-based adjuvant chemotherapy [17-19]. However, a recent study indicates a lack of association with hENT1 expression in the prospective multicenter NCT01124786 trial. The discrepancies observed might be due to the use of different methods, treatments heterogeneity, and relatively small sample size. Standardized techniques of sample collection/processing, larger and uniformly treated populations and integration with functional data, are crucial to validate the best markers for personalized treatment of PDAC patients.

1.4. MicroRNA-211 as a prognostic factor in resected PDAC

The role of RRM2 expression was further investigated in our following studies on microRNAs (miRNAs), since it is one of the targets of miR-211, as described in chapters 5 and 6. Several studies have evaluated the complex genetic networks and transcriptomics alterations underlying the development and progression of PDAC [20,21]. The recent discovery of miRNAs has provided additional insights potentially explaining the gap that exists between tumor genotype and phenotype. MiRNAs play essential roles in the control of proliferation, differentiation and apoptosis, while their aberrant expression in many tumors, indicated that they might function as oncogenes or tumor suppressor genes [22,23], suggesting their use for diagnostic and therapeutic purposes. Moreover, since miRNAs are stable and detectable in human blood they could be useful as diagnostic or prognostic markers. Chapter 5 of this thesis reviews the role of miR-211 in PDAC as well as in other human diseases. The role of this miRNA in PDAC emerged from a comprehensive miRNA expression profiling of more than 1200 human miRNA performed to distinguish resected PDAC patients with short OS (≤12 months) from long term survivors (>30 months), as described in chapter 6. This extensive miRNA microarray analysis resulted in a list of 170 miRNAs that show significant differences in expression between the two groups. RELIEF algorithm and highly stringent statistics identified the top 4 discriminating miRNAs (miR-211, miR-1207-3p, miR-326 and miR-4321) between patients with short- vs. long-OS. We investigated the expression of these miRNAs by RT-PCR in an additional cohort of patients. Patients with low miR-211 expression had a significantly shorter median OS (14.8 months, 95%CI, 13.1–16.5 months) compared to patients with high miR-211 expression (median OS, 25.7 months, 95%CI, 16.2–35.6 months, HR= 3.0, 95%CI, 2.1–8.9, \( P=0.001 \)). Similar results were found for the disease free survival (DFS) of patients with low miR-211 expression, who had a median DFS of 16.7, compared to 9.3 months in patients with high miR-211 expression (\( P=0.004 \)). The Cox proportional hazards regression model used for the multivariate analysis illustrated the prognostic significance of miR-211 expression and
grading. Further in vitro studies were performed on the expression and role of miR-211 in PDAC cells. MiR-211 was expressed in all the PDAC cell lines and primary tumor cell cultures, and its modulation was related to gemcitabine cytotoxicity. Induction of the miR-211 expression in the cells increased the sensitivity to gemcitabine and reduced the expression of its target RRM2.

In conclusion, our studies identified some novel prognostic and predictive markers, including polymorphisms and miRNA expression. However, future studies should validate these candidate markers in larger cohorts, ideally in the prospective and multicenter setting. Randomized studies with a control arm of patients treated with other regimens and the comparison of the survival stratified by miRNA expression would be the only way to establish their predictive role. Moreover, these studies should also implicate the potential use of our candidate biomarkers in the neoadjuvant setting, which might provide an alternative for patients with aggressive disease, who could be given chemotherapy before surgery to kill any micrometastases.

Scope 2: Therapeutic potential of novel anticancer agents in treatment of PDAC

Recent advances in genome sequencing have identified a complex picture of genetic aberrations in the initiation and progression of PDAC [20,21]. These genetic alterations influence the activity of core signaling pathways (e.g., Akt/PI3K and Met/HGF pathways), which are altered in the majority of analyzed PDACs, representing targets for novel therapeutic strategies. Of note, also some of the markers that were evaluated in our previous studies, such as EZH2 (chapter 2), might be targeted by new drugs, as described in chapter 7. Therefore, the main aim of the second part of this thesis was to evaluate new agents for treatment of PDAC, as described in chapters 7-10 and summarized below.

2.1. Inhibition of EZH2 reduces aggressiveness of PDAC and enhances sensitivity to gemcitabine

Recently PDAC emerged as a Cancer Stem Cell (CSC) – driven disease. Pancreatic CSCs are highly tumorigenic and have the abilities to self-renew and produce differentiated progeny. CSCs have also been associated with chemoresistance to gemcitabine [25,27]. Against this background, studies on key determinants in CSCs can provide both biomarkers of the aggressiveness of PDAC and novel targets to overcome chemoresistance. EZH2 is a histone methyltransferase essential for self-renewal of CSCs [28]. In chapter 7, we evaluated the EZH2 expression in PDAC tissues and cells, and investigated the cell growth inhibitory effect of the EZH2 inhibitor DZNeP in combination with gemcitabine in monolayer cell cultures and cells growing as spheroids in serum-free-CSC-medium. EZH2 was expressed in all our PDAC cells, including 7 primary tumor cell cultures, while the expression was significantly lower in both fibroblasts and normal pancreatic ductal cells HPNE. DZNeP significantly reduced EZH2 and H3-K27 expression and we also showed that its combination with gemcitabine was synergistic. This synergistic interaction against cell proliferation was associated with a significant increase in apoptosis induction.

However, our findings showed that this synergistic interaction is also mediated by other mechanisms, which reduced the aggressiveness of PDAC and enhanced sensitivity to gemcitabine. Consistent with previous studies showing that inhibition of EZH2 by DZNeP,
Discussed attenuated glioblastoma and mesothelioma cell migration/invasion [29], our data showed a marked reduction of cell migration. Keeping with previous findings on inverse relationship between EZH2 and E-cadherin expression [10], our data showed that DZNeP-induced EZH2 inhibition resulted in an increase in both mRNA and protein expression of E-cadherin. Moreover, DZNeP significantly reduced the growth of PDAC spheroids in serum-free-stem-cell medium and effectively depleted the most aggressive subpopulation of PDAC cells, as suggested by the significant reduction of CSC-marker CD133 expression. Moreover, we analyzed the expression of key nucleoside transporters (hENT1 and hCNT1), showing that DZNeP significantly increased the expression of these transporters. These findings can be explained at least in part by the reduction of endogenous deoxynucleotides, which might determine the up-regulation of both hENT1 and hCNT1, potentially facilitating gemcitabine cytotoxicity.

In conclusion, inhibitors of EZH2, such as DZNeP, seem promising anticancer agents, by attacking key mechanisms involved in the proliferation, cell cycle control, apoptosis as well as migration properties of PDAC cells. All these molecular mechanisms underlying the synergism of DZNeP/gemcitabine combination, support further studies on this novel therapeutic approach as well as on novel anti-EZH2 compounds, with a better pharmacokinetic profile than DZNeP, to improve the efficacy of the actual treatment of PDAC.

2.2. Inhibition of Akt/PI3K signaling pathway increases the chemosensitivity of PDAC cells to gemcitabine

The Akt/PI3K pathway is one of the core signaling pathways affected in PDAC [20]. Akt is overexpressed in more than 40% of PDAC patients [30,31], and has been shown to be associated with PDAC poor prognosis and chemoresistance [32]. The Akt/PI3K pathway regulates tumor-associated cell processes such as cell growth, cell cycle progression, survival, migration, epithelial–mesenchymal transition (EMT) and angiogenesis [33]. Fahy and colleagues showed that inhibition of the PI3K/Akt pathway sensitizes PDAC cells to the apoptotic effect of PI3K or Akt inhibitor both in vitro and in vivo [33]. Therefore, we investigated the therapeutic potential of the novel Akt inhibitor perifosine in combination with gemcitabine in PDAC cells in chapter 8.

Perifosine is a synthetic alkylphosphocholine that inhibits Akt activation by targeting the pleckstrin homology domain of Akt [34]. Anti-tumor activity of this drug has been observed in a variety of cancers in vitro and in vivo [35] and its clinical efficacy was evaluated in phase II/III clinical trials in patients with advanced solid tumors, including breast and prostate cancers [36,37]. Unfortunately, a phase II study in locally advanced, unresectable, or metastatic PDAC failed, as a result of unacceptable adverse events [38]. Therefore we evaluated the therapeutic potential of this inhibitor in several representative PDAC cells and primary PDAC cells, in order to identify biological factors that can be used to tailor this treatment. We observed that perifosine inhibited cell growth and interact synergistically with gemcitabine in PDAC cells with high expression of Akt, while an antagonist interaction was observed in cells with low Akt expression. The synergistic effect was associated with reduction of the expression of RRM1 and RRM2, potentially facilitating gemcitabine cytotoxicity. Furthermore, this synergistic effect was associated with inhibition of growth of PDAC spheroids. In line with the inverse relationship between Akt and E-
cadherin expression [39], our results showed that perifosine increased the expression of E-cadherin in the PDAC cells.

Since the Akt pathway plays an important role in cell survival process, its blockage can result in activation of programmed cell death [40]. Therefore, we also evaluated the effect of perifosine on cell cycle perturbation and apoptosis induction, showing significant \((P<0.05)\) enhanced apoptosis in PDAC cells with high Akt expression. This was associated with activation of a number of pro-apoptotic markers, including caspase -3/-6/-9, BAD and PARP, and inhibition of NF-kB and Bel-2 expression.

In aggregate, our data provide novel insights in the antitumor activity of perifosine in PDAC, supporting the analysis of the expression of Akt and other biomarkers for the rational development of this therapeutic approach.

2.3. Antitumor activity of novel c-Met/ALK inhibitor crizotinib in PDAC

The receptor tyrosine kinase Mesenchymal-Epithelial Transition factor (c-Met) plays essential roles in embryonic and adult life, including embryonic development, tissue homeostasis and morphogenesis [41-43]. Conversely, abnormal stimulation of this pathway contributes to cellular transformation, epithelial-to-mesenchymal transition, tumor invasion, progression and metastasis [44,45]. The c-Met/HGF signaling pathway is aberrantly activated or overexpressed in a variety of solid tumors, including PDAC [43,46-51]. Of note, c-Met is expressed in the developing pancreatic bud of the embryo and marks candidate stem/progenitor cells in the embryonic and adult pancreas, but it is expressed at very low levels in normal adult differentiated pancreatic cells [52]. However, the \(MET\) gene is amplified or overexpressed in progenitor ductal cells [53] and emerged as a stem cell marker in pancreatic tissues [54] as well as a marker of pancreatic CSC [51]. Moreover, interactions between cancer cells and fibroblasts through c-Met increased PDAC invasiveness [55,56], and factors affecting the cancer/stroma interaction play a key role in PDAC progression and aggressive behavior [57]. Other studies demonstrated that PDAC cells overexpressing c-Met are chemoresistant to gemcitabine [58,59].

Therefore, in chapter 9 of the thesis, we describe the critical role of the HGF/c-Met signalling pathway in upper gastrointestinal cancers, as well as the preclinical and clinical investigations on c-Met inhibitors in solid tumors, with particular emphasis on recent findings with small-molecule inhibitors in PDAC. As summarized in this review, a variety of different strategies to inhibit this signaling pathway have been investigated ([1] inhibition of HGF, and [2] inhibition of Met with Met antibodies or [3] tyrosine kinase (TK) inhibitors), and a large number of new molecules entered preclinical and clinical investigations. Crizotinib has recently been approved for ALK-rearranged non-small cell lung cancer, while the clinical efficacy of tivantinib needs to be ultimately validated in ongoing phase III randomized trials. On the other hand, several important questions remain to be unanswered on the molecular mechanisms underlying the antitumor effects of these drugs, as well as on their possible role in combination treatments of different tumor types, including PDAC.

Using the knowledge gained from the chapter 9, the therapeutic potential of the c-Met/ALK TK inhibitor crizotinib was tested alone and in combination with gemcitabine in PDAC cells including the Capan-1-gemcitabine-resistant cells (Capan-1-R), as described in chapter 10.
Recently, pancreatic CSCs have been shown to be associated with the aggressiveness of PDAC, metastatic behavior and intrinsic resistance to chemotherapy [51,60]. Li and colleagues identified a subpopulation of highly tumorigenic cancer cells expressing the cell surface markers CD44, CD24, and CD326 [61]. Consistent with these findings, our results illustrated that the expression of CD44, CD24 and CD326 were increased at the mRNA and protein levels in Capan-1-R compared to Capan-1 cells. Moreover, these cells have higher expression of c-Met and phospho-c-Met, suggesting that the c-Met signaling pathway could be a valuable target to overcome chemoresistance. Therefore, we evaluated the pharmacological interaction of crizotinib with gemcitabine, showing that this combination was synergistic. In addition, crizotinib down-regulated the expression of CD44+/CD133+/CD326+, as well as that of c-Met.

We also evaluated the expression of EMT markers showing that levels of E-cadherin were not affected, while vimentin expression was increased in Capan-1-gemcitabine-resistant cells compared to Capan-1 cells. These results are in agreement with a previous study, showing that gemcitabine-resistant cells were more invasive and migratory and had increased vimentin expression [62]. Notably, crizotinib significantly reduced vimentin expression, resulting in an impaired migration, which might be attributed, at least in part, to a reversal of their EMT phenotype.

Finally, we investigated the expression of the main determinants of gemcitabine activity in Capan-1-R and Capan-1 cells, as well as the effect of crizotinib on the modulation of these genes. These results showed that the mRNA expression of hCNT1, deoxycytidine kinase and RRM1 and RRM2 were significantly reduced in Capan-1-R cells compared to Capan-1. Conversely, the mRNA expression and enzyme activity of cytidine deaminase (CDA) in Capan-1-R cells were significantly higher than in Capan-1 cells. This can potentially explain the significantly lower levels of gemcitabine metabolites in Capan-1-R compared to Capan-1. Moreover, crizotinib markedly decreased the CDA expression and increased hCNT1 expression, potentially reducing gemcitabine catabolism, while increasing gemcitabine uptake.

In conclusion, our findings provide novel insights into the antitumor activity of crizotinib in PDAC cells, unraveling its ability to specifically target CSC-like-subpopulations, interfere with cell-proliferation, induce apoptosis, reduce migration and synergistically interact with gemcitabine, supporting further studies on this novel therapeutic approach for PDAC. Therefore, we further explored the therapeutic potential of this therapeutic regimen in orthotopic PDAC mouse models derived from primary PDAC cells, as described in the Chapter 11.

**Scope 3: Development of new patient-derived orthotopic mouse PDAC models: Towards new treatment strategies**

As discussed above, some of the unfavorable clinical factors, which may have at least in part, hampered clinical progress in PDAC include: (1) selection of most anticancer drugs in clinical trials on the basis of their activity in preclinical models that do not recapitulate the complex molecular and histopathological hallmarks of PDAC, and (2) lack of appropriate drugs that combine synergistically with gemcitabine.

In the context of preclinical models, the most commonly used PDAC models include 1) established cell line xenografts cultured as monolayers or grown as their xenografts, and 2)
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3.1. Crizotinib inhibits metabolic inactivation of gemcitabine in c-Met-driven pancreatic carcinoma

We have established 4 primary PDAC cell cultures (PDAC-1/-2/-3/-4) from surgically resected tumor masses using sterile non-necrotic tumor samples (40% efficiency). These cells were successfully co-transduced with two lentiviral vectors, expressing Fluc-mCherry (FM) and Gluc-CFP (GC) and then injected orthotopically into the pancreas of three athymic mice. All four primary transduced PDAC cell cultures engrafted and developed tumors in all the mice injected (100% take rate) and expanded over time, as determined by the increase in Fluc and Gluc signal intensities. Remarkably, survival trend observed in the mice followed a same trend in the PDAC patients, although the number of models does not warrant a statistically supported survival correlation. Moreover, MR-scans confirmed the localization of tumor cells in the pancreas, as well as retroperitoneal invasion, while high-frequency-ultrasound enabled for the measurement of tumor volumes and revealed hypovascular pancreatic tumors.

All these models showed the main histopathological features of human PDAC, compared to surgical material resected from the human originator tumor, including tumor infiltration, stroma and PDAC-associated desmoplastic reaction, ductal characteristics, adenocarcinoma differentiation, and inflammation. In addition, the xenografts stained positively with human specific antibodies, which are routinely used in PDAC immunohistochemistry. Moreover, we evaluated the hypovascular and hypoxic areas by analysis of CD31 and Carbonic
Anhydrase IX (CAIX), respectively [72,73]. These results showed that the blood vessels were organized in the stroma surrounding the tumor nests and did not invade into the tumor. Similarly, most tumors showed high expression of the hypoxia marker CAIX. Importantly, most of the orthotopic tumors metastasized to other organs and followed the typical routes of invasion to lymph nodes, liver and lung, as observed in patients.

After finding a similar phenotype, we investigated whether the genetic signature of the human originator tumors was preserved. The orthotopic PDAC models showed similar genomic abnormalities as their human originator tumors, and genotypic heterogeneity was reflected by an average of more than 50 different aberrations, as detected by high-resolution array comparative genomic hybridization (aCGH) and mutation studies. These models can therefore be used as reliable tools for understanding the role of complex PDAC genetic characteristics and testing new targeted drugs.

Interestingly, in one of our PDAC specimens, PDAC-3, we observed a high copy number gain of the c-MET gene. These results were validated by copy number analysis as well as by gene and protein expressions analysis. Then we tested the activity of three novel c-Met inhibitors, tivatinib, crizotinib and DN-30. All these compounds were more effective in the PDAC-3 cells compared to other PDAC cells, and crizotinib emerged as the most active inhibitor of cell growth. The cells were also treated with the combination of crizotinib and gemcitabine, median drug-effect analysis showed strong synergism in these cells, in parallel with increase in the accumulation of gemcitabine-nucleotides. Since previous studies suggested that gemcitabine is inactivated by CDA [74,75], we investigated CDA activity in the cells upon treatment with crizotinib, showing a significant reduction in CDA activity. This reduction was explained by the degradation mediated by crizotinib-induced ROS, as determined by analysis of reactive oxygen species (ROS) activity. This might at least in part explain the synergistic effect.

Furthermore, crizotinib significantly reduced tumor growth in PDAC-3-FM-GC mice, which survived longer than untreated controls. Moreover, liquid chromatography-tandem mass spectrometry analyses demonstrated significantly higher concentrations of gemcitabine in the tumors and blood samples from mice treated with the gemcitabine-crizotinib combination compared to gemcitabine alone.

In conclusion, with the establishment and extensive genetic and histopathological characterization of our double bioluminescent patient-derived orthotopic mouse PDAC models, we provided novel preclinical models to explore therapeutic strategies and mechanistic insights that can ultimately be applied to the future clinical practice for the individualized treatment of PDAC patients. Here we used our models to identify crizotinib and gemcitabine as a promising drug combination, acting synergistically via simultaneous targeting of key intratumoral genetic features and increase of drug delivery by CDA modulation, and warranting further investigation for the treatment of PDAC.

3.2. CYB5A as a novel prognostic factor for PDAC, exerting its tumor-suppressor function via autophagy-induction and TRAF6 modulation

Our models were also used in a study aiming at the identification of novel prognostic biomarkers. A recent study showed the correlation of genomic imbalances and clinical outcome using aCGH in 44 radically resected patients [76] (Lee et al., 2012), demonstrating a significant association between shorter survival and loss of the small cytoband 18q22.3.
Therefore, in the chapter 12, we further evaluated whether the mRNAs and/or proteins encoded by the genes in the 18q22.3 cytoband were associated with the outcome of PDAC patients in two homogeneous cohorts of radically resected patients. Moreover, we explored the role of CYB5A in primary tumor growth and metastatic spread using an orthotopic PDAC mouse model.

The genes located in the 18q22.3 cytoband (FBXO15, C18orf55, CYB5A, CPGL, and CPGL-B transcripts) were detectable in most samples, however median expression values of CYB5A and C18orf55 were significantly lower in deleted versus non-deleted samples. Moreover, patients with tumors expressing low levels of the CYB5A gene had a shorter OS than the high expression group (HR=2.3, P=0.01). Similar results were observed for the DFS curves. The prognostic role of CYB5A was then validated by IHC analysis of specimens from an independent cohort of 100 radically resected PDAC patients, collected in a tissue-microarray. The multivariate analyses demonstrated that CYB5A expression was an independent prognostic factor, and lower expression levels of CYB5A were correlated with an increased risk of death (HR=2.0, P=0.02), and relapse (HR=1.8, P=0.03).

Then we investigated the role of CYB5A in PDAC biology; we initially assessed the CYB5A expression in 11 PDAC cell lines, 5 primary cell cultures and in the immortalized normal ductal epithelial HPNE cells, showing lower expression levels of CYB5A in all of the malignant cells, compared to the non-tumorigenic control. Su86.86 and PDAC-2 cells were selected for further studies, as they both carried the 18q22.3 cytoband loss. We successfully established CYB5A overexpressing stable subclones and empty vectors (named CYB5A+, and CTR respectively). We demonstrated that CYB5A retrovirus-mediated up-regulation reduced proliferative and invasive capacity while increasing autophagy induction, as determined by electron microscopy. Furthermore, in order to shed light on the molecular events driving autophagy, we performed a PCR array on 84 key autophagic genes, revealing a significant up-regulation of BAX, accompanied by down-regulation of BCL-2 and MAPK14, while a kinase array revealed the down-regulation of phospho-EGFR and phospho-MAPK14. Network analyses suggested the interaction of CYB5A with TRAF6, a molecular bridge for many diverse signals, both upstream and downstream, including Akt and MAP kinases, as well as with several genes involved in cell death. Both transduced PDAC-2- and Su86.86 cells had a marked reduction of TRAF6 levels, which was restored in a rescue experiment with the transfection of CYB5A siRNA.

Finally, we tested the tumor-suppressor function of CYB5A in our orthotopic mouse models derived from the CYB5A+ retrovirus-transduced cells, which had increased survival, as well as reduced primary tumor growth and metastatic spread. Moreover, the tumors in the CYB5A+ group had a weak expression of TRAF6 with respect to the moderate/strong staining in the CTR group.

In conclusion, we identified CYB5A as a novel inhibitor of anti-autophagy oncogenic phenotypes, raising the possibility at restoring CYB5A activity, via gene therapy, or targeted therapy inhibiting its potential downstream TRAF6. This might constitute a novel approach that interferes with multiple signalling components of EGFR, TGF-β, Akt and Src, favouring cancer cell death while preventing potentially deleterious cross-talk between these pathways in specific subgroups of PDAC patients.
Conclusions and Future Directions

The studies described in this thesis show that several genetic and epigenetic alterations are the main driving forces of PDAC progression and metastasis. Understanding these alterations is the first step on the road to improve the current outcome. The signaling pathway affected by these alterations should be the target for tailored treatment of these patients. In particular, here we demonstrate that c-Met/HGF and Akt/PI3K pathways constitute potential important targets. Targeting the stromal compartment of the tumor is another new promising strategy that may improve the poor prognosis of PDAC. Further investigations are needed to identify and select the optimal patient populations that will benefit from specific treatments. However we reckon that our investigations could provide innovative tools for further progressing in treatment of this disease.

It is obvious that there is still a long way to go for the finding a cure for this devastating disease. For future years, it is hoped that insights from preclinical and translational studies will result in improvement of treatment and survival and hopefully the results of the research described in the current thesis contribute to that.
References


