Discussion
Chapter 8

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Discussion

Pancreatic cancer is the fourth leading cause of cancer death in developed countries [1]. There are approximately 277,000 new cases of pancreatic cancer and 266,000 deaths annually worldwide, indicating a mortality rate of 96% of the cases diagnosed [2-3]. Despite extensive clinical and scientific researches, the prognosis of this disease has not improved over the past decades [4]. The main reasons for this poor prognosis are invasive behavior and resistance to currently available chemotherapy regimens [5]. Following the drug’s approval in 1997, gemcitabine became the standard of care for patients with advanced disease. The PRODIGE 4/ACCORD 11 trial established the efficacy of the leucovorin/fluorouracil/irinotecan/oxaliplatin (FOLFIRINOX) combination regimen in patients with previously untreated metastatic pancreatic adenocarcinoma based on a significant improvement in median overall survival over gemcitabine (11.1 vs. 6.8 months; HR 0.57, 95% CI [0.45, 0.73]; p < 0.0001. (6) Despite this survival benefit, enthusiasm for FOLFIRINOX was tempered by increased toxicity in comparison with gemcitabine, particularly vomiting (14.5% vs. 4.7%; p = 0.002), diarrhea (12.7% vs. 1.2%; p = 0.0001), thrombocytopenia (9.1% vs. 2.4%; p= 0.008), peripheral neuropathy (9.0% vs. 0%; p = 0.001), and febrile neutropenia (5.4% vs. 0.6%; p = 0.009). Similarly, recent results of the phase III MPACT trial established the benefit of combination therapy with nab-paclitaxel plus gemcitabine over gemcitabine alone as first-line treatment of metastatic pancreatic adenocarcinoma. In particular, median overall survival increased from 6.7 months with gemcitabine alone to 8.5 months with the combination (HR 0.72, 95% CI [0.617, 0.835]; p < 0.001). However, nab-paclitaxel/gemcitabine was more toxic than gemcitabine. The most common grade 3 toxicities were neutropenia (38% in the nab-paclitaxel-gemcitabine group vs. 27% in the gemcitabine group), fatigue (17% in the nab-paclitaxel/gemcitabine group vs. 1% in the gemcitabine group), and neuropathy (17% in the nab-paclitaxel/gemcitabine group vs. 1% in the gemcitabine group) (7). Therefore, gemcitabine monotherapy is still the standard treatment for most advanced PDAC. Recently, gemcitabine has also become the mainstay of adjuvant therapy in PDAC, leading to a 24% improvement in overall survival, with a statistically significant absolute 10.3% improvement in the 5-year overall survival rate (20.7% vs 10.4%) and a 4.5% improvement in the 10-year survival rate (12.2% vs 7.7%), compared with observation alone [8]. However, the prognosis for PDAC patients remains extremely poor, and for all stages combined the 1-year relative survival rate is 25%, while the 5-year survival is estimated as less than 5% [1]. Therefore, there is an urgent need for new therapeutic options in PDAC. In the past years it became evident that deregulation of epigenetic mechanisms and metabolism plays an important role in pancreatic carcinogenesis. The research described in the current thesis was mainly focused on the exploitation of these mechanisms as (1) new biomarkers and (2) promising targets for therapeutic interventions, as they play a pivotal role in the regulation of key cellular processes in PDAC cells proliferation and invasion.
Scope 1: Genetics and epigenetics factors influencing PDAC prognosis

1.1. 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 [9]. Recently, other molecular events, such as epigenetic alterations were identified to contribute to the development and progression of PDAC.

In particular, the epigenetic gatekeeper Enhancer of Zeste Homolog 2 (EZH2) is becoming increasingly acknowledged as a prognostic biomarker in radically resected PDAC patients [10]. Therefore in chapter 2, we evaluated the prognostic value of EZH2 in 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 [11-12], 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, 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.

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 (i.e. miR-101), which could be investigated in enriched tumor-derived exosomes in peripheral blood [13], should be evaluated in order to improve the clinical management of advanced PDAC patients.

1.2 MicroRNA-211 is a prognostic factor in resected PDAC, and modulates PDAC cells invasive behavior and gemcitabine activity

Epigenetics has recently been extended to microRNAs (miRNA). Several studies have evaluated the complex genetic networks and transcriptomics alterations underlying the development and progression of PDAC [14-15], and miRNA could provide additional insights potentially explaining the gap between tumor genotype and phenotype. Indeed, miRNAs play essential roles in the control of proliferation, differentiation and apoptosis,
and their aberrant expression support their function as oncogenes or tumor suppressor genes [13].

Research to explore the miRNA expression signature that is associated with PDAC is increasing, and several candidate miRNAs for diagnostic, prognostic and therapeutic purposes have been identified. Chapter 3 of this thesis reviews the role of miR-211 in PDAC as well as in other human diseases. In our previous study [16], the prognostic role of this miRNA emerged from a comprehensive miRNA expression profiling of more than 1200 human miRNA, which was performed to distinguish resected PDAC patients with short OS (≤12 months) from long term survivors (>30 months). Therefore, in chapter 4 of the current thesis we further investigated the role of miR-211 in gemcitabine activity in PDAC cells with different migratory abilities. Moreover, we evaluated whether miR-211 might affect gemcitabine chemosensitivity. Our findings demonstrate that enforced expression of miR-211 with pre-miR-211 is associated with inhibition of cell proliferation, as well as with reduction of migration and invasion of PDAC cells. Moreover, the present study supports the possible role of RRM2 as a target of miR-211, which might explain the reduced sensitivity to gemcitabine in cells transfected with anti-miR-211.

Scope 2: Targeting key signaling pathway and fuel supply in pancreatic cancer

Recent advances in genome sequencing have defined PDAC as a cancer with alterations of a wide range of signaling cascades, which is in contrast with certain tumors that are driven by a single targetable oncogene However, certain signaling pathways (e.g., Wnt/beta-catenin and PI3K/Akt pathways), and key nodal points act as core genetic alterations commonly detected in PDAC cells [14-15], representing optimal targets for novel therapeutic strategies.

In addition, pancreatic cancer is characterized by a metabolic-switch to anaerobic-glycolysis and alteration in the activation and expression of metabolic enzymes such as lactate-dehydrogenase (LDH-A), which is associated with cell growth, metastasis and chemoresistance. Therefore, the main aim of the second part of this thesis was to evaluate new agents for treatment of PDAC, as described in the chapters 5-7.

2.1. Modulation of galectin-4 and Wnt/β-catenin pathway inhibit the invasive behavior and proliferation of PDAC cells

A previous study demonstrated that galectin-4 (Gal-4) was overexpressed in more differentiated PDAC cell lines, compared to those having higher metastasis properties [17]. Galectins play an essential role in cell-cell/matrix adhesion and growth regulation [17-18], and it has been shown that galectins are aberrantly expressed in human cancers and associated with cancer initiation, progression [19-20] or cancer-associated stromal cells [21]. Furthermore, Satelli and colleagues showed that Gal-4 is down-regulated in invasive colorectal cancer [22], whereas the enforced expression of Gal-4 in colorectal cancer cells inhibited the Wnt/β-catenin pathway, suggesting its role as a tumor suppressor gene [22]. Importantly, the activation of Wnt/β-catenin pathway is required for initiation and progression of PDAC [23]. Thus, inhibition of Wnt/β-catenin pathway in cancer cells selected on the basis of Gal-4 expression, might have a potential therapeutic impact on suppression of tumor progression.
In chapter 5, we explored the expression of Gal-4 in PDAC tissues, as well as the role of Gal-4 on invasive behavior and regulation of Wnt/β-catenin pathway in several representative primary PDAC cell cultures and xenografts. The analysis of Gal-4 expression in human PDAC tissues showed that ≈80% of patients without lymph node metastasis had a high expression of Gal-4, while 70% of patients with metastasis to lymph nodes had a low Gal-4 expression. Moreover, Gal-4 was differentially expressed in human primary PDAC cells, and primary PDAC cells with high expression of Gal-4 showed less migratory or invasive ability in vitro and in vivo. Conversely, knockdown of Gal-4 significantly increased invasion and migration.

Finally, enforced expression of Gal-4 markedly reduced the expression of β-catenin and increased the sensitivity to the Wnt/β-catenin inhibitor ICG-001, providing novel insights into the therapeutic potential of Gal-4 and its cross talk with Wnt/β-catenin signaling pathway in PDAC.

2.2. Inhibition of Akt/PI3K signaling pathway in cells with phospho-Akt overexpression increases the chemosensitivity of PDAC cells to gemcitabine

The Akt/PI3K pathway is one of the core signaling pathways affected in PDAC [14]. Akt is overexpressed in more than 40% of PDAC patients [24-25], and has been shown to be associated with PDAC poor prognosis and chemoresistance [26]. The Akt/PI3K pathway regulates tumor-associated cell processes such as cell growth, cell cycle progression, survival, migration, epithelial–mesenchymal transition (EMT) and angiogenesis [27]. 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 [28]. Therefore, we investigated the therapeutic potential of the novel Akt inhibitor perifosine in combination with gemcitabine in PDAC cells in chapter 6.

Perifosine is a synthetic alkylphosphocholine that inhibits Akt activation by targeting the pleckstrin homology domain of Akt [29]. Anti-tumor activity of this drug has been observed in a variety of cancers in vitro and in vivo [30-31] and its clinical efficacy was evaluated in phase II/III clinical trials in patients with advanced solid tumors [32]. Unfortunately, a phase II study in unselected locally advanced or metastatic PDAC patients failed [33]. 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 interacted synergistically with gemcitabine in PDAC cells with high expression of Akt, while an antagonistic 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, in line with the inverse relationship between Akt and E-cadherin expression [34], our results showed that perifosine increased the expression of E-cadherin in the PDAC cells, reducing their invasive behavior.

Since the Akt pathway plays an important role in cell survival process, its blockage can result in activation of programmed cell death [35]. 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-κB and Bel-2 expression.
In conclusion, our findings provide novel insights in the antitumor activity of perifosine/gemcitabine combination in PDAC, supporting the analysis of the expression of Akt and other key biomarkers for the rational development of this therapeutic approach.

2.3. Inhibition of LDH-A reduces PDAC aggressiveness and enhances sensitivity to gemcitabine

Pancreatic cancer is characterized by a dense desmoplastic stroma and a sparse vascularization that limit oxygen/nutrients availability. This architecture induces severe hypoxic stress to tumor cells, which become resistant to chemotherapy and are able to increase their invasive and metastatic potential [36-37]. The ability of cancer cells to survive under hypoxic conditions results from their ability to reprogram canonical biochemical pathways in order to provide sufficient energy [38-39]. A recent study by Zhou and colleagues showed several metabolic changes in proteomes of PDAC cells compared to normal pancreatic duct cells [40]. As a result of these metabolic changes, cancer cells tend to take up more glucose and generate lactate through LDH-A [41].

The knockdown of LDH-A by shRNA stimulated mitochondrial oxygen-dependent oxidative phosphorylation, decreased cell proliferation under hypoxia, suppressed tumorigenicity and enhanced chemosensitivity [40, 42-43]. Of note, the inhibition of LDH-A can be achieved without influencing the energy balance of normal tissues [43], and has not been shown to raise major side effects, in agreement with the observation that hereditary LDH-A deficiency does not provoke any symptoms under ordinary circumstances in humans [45]. All these findings suggest that LDH-A is a promising and safe target, and novel LDH-inhibitors represent an innovative, selective and also potentially nontoxic strategy of interfering with hypoxic cancer growth.

Recently, we developed a new class of N-hydroxyindole-based inhibitors of LDH-A (NHI), which proved to inhibit the growth of different tumor cells, including PDAC cells, especially in hypoxic conditions [45-46]. Therefore in chapter 7, we evaluated the molecular mechanisms underlying the inhibition of LDH-A, using two of the most promising compounds among our new LDH-A inhibitors. The compounds were investigated in combination with gemcitabine in PDAC cells in normal and hypoxic conditions. For this purposes we determined the LDH-A expression in 15 PDAC cells, including 7 primary tumor cell cultures, where the levels of LDH-A mRNA were significantly higher in PDAC cells, compared to normal pancreatic ductal cells HPNE. The expression of LDH-A was significantly increased under hypoxic conditions, which was associated with increased LDH-A activity. NHI inhibitors of LDH-A were effective against PDAC cells under hypoxic condition and their combination with gemcitabine was synergistic in PDAC cells under normoxic and hypoxic conditions. Moreover, the inhibition of CDA makes these compounds excellent candidates for combination with gemcitabine, and this synergistic effect was associated with modulation of key mechanisms involved in cell proliferation, cell cycle control, apoptosis, stemness, and migration properties of PDAC cells, especially under hypoxic conditions.
Conclusions and Future Directions

The studies described in the current thesis demonstrate that several genetic, epigenetic and metabolic alterations are the main driving forces of PDAC progression and metastasis. Identification and understanding of these changes is the first step on the road to identify novel biomarkers and targets to improve the treatment and outcome of PDAC.

However, all the targets and effects of drugs affecting epigenetics and metabolic aberrations are not fully elucidated yet. For example new miRNAs and new targets of known miRNAs are continuously discovered. Moreover, miRNA targeting not only affects pathways involved in carcinogenesis but also normal pathways leading to unwanted side effects. Similarly, although epigenetic drugs such as inhibitors of EZH2 are regarded as targeted therapies, they might affect many different pathways and tissues by exerting their effect in both tumor and normal cells.

Our studies provide innovative ideas and tools for further progress in treatment of PDAC. However, further investigations to validate our findings and establish all downstream effects of epigenetic and metabolic targeting are warranted. This will provide a basis for understanding the cause of adverse effects or drug resistance, and can ultimately lead to optimal drug regimens, with appropriate patients selection in PDAC.

In conclusion, it is hoped that insights from future preclinical and translational studies will result in improvement of treatment and survival of PDAC patients, and hopefully the results of the research described in the current thesis contribute to that.
References

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