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Molecular and genetic bases of pancreatic cancer

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Abstract

Pancreatic cancer remains a formidable challenge for oncologists and patients alike. Despite intensive efforts, attempts at improving survival in the past 15 years, particularly in advanced disease, have failed. This is true even with the introduction of molecularly targeted agents, chosen on the basis of their action on pathways that were supposedly important in pancreatic cancer development and progression: indeed, with the notable exception of the epidermal growth factor receptor (EGFR) inhibitor erlotinib, that has provided a minimal survival improvement when added to gemcitabine, other agents targeting EGFR, matrix metallo-proteases, farnesyl transferase, or vascular endothelial growth factor have not succeeded in improving outcomes over standard gemcitabine monotherapy for a variety of different reasons. However, recent developments in the molecular epidemiology of pancreatic cancer and an ever evolving understanding of the molecular mechanisms underlying pancreatic cancer initiation and progression raise renewed hope to find novel, relevant therapeutic targets that could be pursued in the clinical setting. In this review we focus on molecular epidemiology of pancreatic cancer, epithelial-to-mesenchymal transition and its influence on sensitivity to EGFR-targeted approaches, apoptotic pathways, hypoxia-related pathways, developmental pathways (such as the hedgehog and Notch pathways), and proteomic analysis as keys to a better understanding of pancreatic cancer biology and, most importantly, as a source of novel molecular targets to be exploited therapeutically.

Introduction.

Pancreatic cancer is the 8th and 9th leading cause of cancer death in men and women, respectively, worldwide and the 4th leading cause of cancer death in Western countries [1, 2]. With mortality closely approaching incidence (>40.000 estimated new cases and approximately 37.000 estimated deaths in 2010 in the US alone) and a 5-yr survival rate of 5% overall [1], pancreatic cancer (and its most aggressive and frequent histological subtype pancreatic ductal adenocarcinoma - PDAC) is arguably the deadliest of solid tumors. Moreover, progresses in PDAC treatment, especially in the advanced disease setting, have been few and modest over the past 20 years [3, 4], resulting in no significant improvement in 5- and 10-yr survival expectations between 1998 and 2003 [5]. Such a dismal picture recognizes a variety of causes, from the lack of early biomarkers and cost/effective screening methods, resulting in diagnosis at late stages of the disease, not amenable to curative surgery [4, 1], to the inherent aggressiveness and resistance to current therapeutic strategies of PDAC cells, particularly in their physiologic environment where they are surrounded by a dense, desmoplastic stroma which supports their growth and dissemination and protects them from conventional, and perhaps even from molecularly targeted, therapeutic agents [4, 6, 7]. In addition, although a core set of common genetic alterations that drive the transformation of pancreatic ductal (or acinar) cells and the progression from precursor lesions (pancreatic intraepithelial neoplasia – PanIN) to frankly invasive, disseminated PDAC have been precisely mapped [8, 9, 10], recent data depict a much more complex genetic/molecular landscape. Indeed, a recent comprehensive genetic analysis of 24 pancreatic cancers identified an average of 63 genetic abnormalities per tumor, that could be organized in 12 different functional, cancer-relevant pathways; moreover, key mutations in each pathway appeared to differ from one tumor to another [11].

In this review, we analyze the results of clinical trials that have been conducted so far with molecularly targeted agents in PDAC, speculating on possible reasons for their failure to improve survival in advanced disease, and provide an overview of recent progresses in the understanding of the molecular bases of pancreatic cancer, with a focus on novel potential targets for therapy.

Targeted therapies for pancreatic cancer: current status and meta-analysis of randomized trials.

We have recently reported on the impact of combination therapy, including regimens employing molecularly targeted agents, on survival of advanced, inoperable PDAC patients [3]. In order to explore the impact of agents targeting specific molecular pathways in such disease setting, we updated our previous meta-analysis including all fully published, phase III, prospective, randomized clinical trials enrolling untreated patients with advanced PDAC, who were randomized to receive a targeted agent in addition to standard gemcitabine monotherapy (TA-G, investigational arm) or gemcitabine alone (G, comparator arm). The analysis was conducted using established methodology[3] to identify significant differences in primary (overall survival - OS) and secondary outcomes (progression-free survival – PFS – and overall response rate – ORR) between investigational and comparator arms. Relative risk (RR) was chosen as measure parameter instead of Peto's odds ratio in order to decrease the risk of overestimation[12]. Six phase III trials were selected, accounting for 3,463 patients. A sensitivity analysis according to the specific molecular pathway targeted by the agent added to gemcitabine (epidermal growth factor receptor – EGFR, farnesyl transferase - FT, matrix metalloproteases – MMP, and vascular endothelial growth factor – VEGF) was conducted, in order to screen for potentially significant differential effects. Overall, no significant OS advantage for TA-G over G was found, in either the overall or the sensitivity analysis (interaction p=0.82, Figure 1 and Table 1); similarly, no differential effects of the pathway inhibitors tested were found in the secondary end-points (Table 1).

Other trials testing novel targeted agents (or combination thereof) in addition to gemcitabine not matching our meta-analysis primary search criteria (see above) have been reported. The AViTA trial [13] investigated, in a randomized phase III setting, the impact of double VEGF/EGFR pathway blockade (using the anti-VEGF mAb bevacizumab and the EGFR tyrosine kinase inhibitor erlotinib) in addition to gemcitabine, as compared to the standard combination of gemcitabine and erlotinib, which had previously shown to modestly, but significantly, prolong survival over gemcitabine alone [14]. With a total of 306 patients randomly assigned

to bevacizumab/erlotinib/gemcitabine and 301 patients assigned to erlotinib/gemcitabine/placebo, the study failed to demonstrate a significant improvement in OS (median OS 7.1 and 6.0 months, respectively; hazard ratio [HR]: 0.89; 95% CI, 0.74 to 1.07; p=0.2087), even though the addition of bevacizumab to erlotinib/gemcitabine significantly improved PFS (HR: 0.73; 95% CI, 0.61 to 0.86; p=0.0002) [15]. Another agent that has been tested in combination with gemcitabine in advanced PDAC is axitinib, a potent oral inhibitor of VEGF receptors 1, 2, 3 [16]. Such agent showed promise in a phase II randomized study [17] in 103 patients with advanced PDAC who were randomly assigned to gemcitabine ± axitinib in a 2:1 ratio, with patients on the axitinib/gemcitabine arm experiencing longer median OS as compared to patients receiving gemcitabine only (6.9 months, 95% CI 5.3−10.1 months, versus 5.6 months, 95% CI 3.9−8.8 months, respectively; HR: 0.71, 95% CI 0.44−1.13). However, such promising results did not hold up in a phase III trial testing the superiority of adding axitinib to gemcitabine versus gemcitabine alone [18]; indeed, based on an interim analysis performed after 630 patients had been accrued and 223 deaths had occurred, the Independent Data Monitoring Committee found no evidence of improvement in the primary endpoint of OS in patients treated with axitinib/gemcitabine as compared to gemcitabine alone and recommended study discontinuation.

Analysis of available data clearly speaks to the failure of targeted agents employed so far to improve outcomes in advanced PDAC. Rather than fostering therapeutic nihilism, these results call for an in-depth analysis of potential reasons for failure. The first reason may be the choice of agents, such as MMP inhibitors, that are largely ineffective as single-agents; indeed, combined analysis of the two randomized studies head-to-head comparing MMP inhibitors with gemcitabine [19, 20], uniformly demonstrates a significantly worse outcome for patients allocated to experimental treatment (RR for OS and ORR 1.45, 95% CI 1.01-2.10, p=0.04, and 0.14, 95% CI 0.05-0.40, p<0.0001, respectively). Second, the chosen agent may fail to hit its putative target(s); this appears to be the case for FT inhibitors, that may fail to effectively block KRAS activity, due alternative activation pathways, such as geranyl-geranylation [21]. General methodological issues, such as insufficient target validation, inadequate phase II testing, and a rush to bring

new compounds into phase III trials [22, 23], may also have contributed to the general failure of new approaches to impact on survival of PDAC patients.

Hints from genetics of hereditary forms and molecular risk factors.

In contrast to other malignancies, such as kidney cancer [24, 25], for which genetic/molecular analysis of hereditary forms has provided useful insights into the pathogenetic mechanisms of sporadic cases as well as an indication of molecular targets to be pursued for therapy, familial cancer syndromes with increased risk of developing pancreatic cancer shed little light on molecular mechanisms of PDAC development and provide little guidance for selecting novel targets for therapy (Table 2) [26, 27]. In approximately 8% of cases PDAC occurs in patients with a family history of pancreatic cancer (i.e. at least two first-degree relatives affected by the disease), the genetic/environmental causes of which remain unknown in most of the cases; however, some cases of familial pancreatic cancer arise in a well-defined, genetically determined cancer predisposition syndrome. Almost all of these syndromes are linked to the failure of DNA damage repair or cell-cycle control mechanisms, functions that are required by all cells, resulting in increased lifetime risk of many different cancers, without any specificity for PDAC (Table 2) [26, 27]. One notable exception is the hereditary pancreatitis syndrome characterized by recurrent attacks of pancreatitis with typical childhood onset and long-term exocrine and endocrine failure; approximately 80% of cases of hereditary pancreatitis are caused by mutations in the cationic trypsinogen (PRSS1) gene, inherited in an autosomal dominant fashion, and carry a specifically increased lifetime risk for pancreatic cancer (up to 53-fold). This risk is correlated with the duration and severity of pancreatitis attacks, with those having early onset of pancreatitis and long-term progression to diabetes being at greatest risk; in addition, genetically determined risk may also interact with environmental factors, as smokers with PRSS1 mutations tend to develop cancer 20 years prior to non-smokers [26, 27]. This situation actually recapitulates the known relationship between environmental risk factors (alcohol intake, smoking habit), pancreas-directed chronic inflammation (chronic pancreatitis), and pancreatic carcinogenesis as it is observed epidemiologically in

sporadic cases of PDAC [28, 27] and may yield novel targets for PDAC therapy, particularly along inflammatory pathways such as cyclooxygenase-2 (COX-2), peroxisome proliferator-activated receptor γ (PPARγ), and nuclear factor κB (NF-κB, reviewed elsewhere in this issue) [29]. Another interesting genetic link between chronic pancreatitis and the development of PDAC is provided by mutations in the SPINK1 gene, which may be inherited and amplify the risk of pancreatitis due to environmental alcohol exposure and have recently been shown to occur in a high proportion of cases of PDAC developing in a chronic pancreatitis background and, at much lower frequency, in sporadic PDAC cases [28, 26, 30].

Finally, insights into putative novel targets for therapeutic intervention in PDAC come from the molecular dissection of the epidemiological/clinical relationships between obesity, diabetes, and pancreatic cancer (recently reviewed in ref. [31]). Both obesity [32, 33, 34] and diabetes [35, 36, 37] are established risk factors for pancreatic cancer and obesity may also impact on PDAC-specific survival [34, 31]. Although the molecular mechanisms underlying such epidemiological associations remain to be established, an emerging view links obesity and diabetes to PDAC development and progression through a complex interaction between insulin, the insulin-like growth factor-1 (IGF-1)/IGF-1R system, and adipokines [31]. This view is supported by recent evidence from genome-wide association studies identifying the nuclear receptor 5A2 (*NR5A2*) gene as a significant predisposing factor for PDAC [38]; interestingly NR5A2 has also been implicated in the development of diabetes and is associated with obesity through its transcriptional control of the adiponectin gene, thus providing a possible molecular link between metabolic dysfunction and PDAC development [39, 40]. Another epidemiological finding that might open the way to novel mechanistic insights into PDAC development, and potentially yield novel therapeutic targets, is the association between genetic variations in the ABO blood group locus and pancreatic cancer risk [41, 42], which may be explained mechanistically by the contribution of the ABO system to the regulation of inflammation and thrombosis [31].

EMT program and EGFR pathway in pancreatic cancer.

Increasing evidence indicates that cancer cells are subjected to the epithelial-mesenchymal transition (EMT), a process by which epithelial cells undergo remarkable morphologic changes characterized by a transition from an epithelial to a fibroblastic phenotype (mesenchymal phenotype) leading to increased motility and invasion [43, 44]. The process of EMT involves loss of epithelial cell-cell junction, actin cytoskeleton reorganization, and up-regulation of mesenchymal molecular markers such as fibronectin, α-smooth muscle actin (SMA), vimentin, and N-cadherin. The zinc finger Snail homologues (Snail1, Snail2/Slug, and Snail3) and several basic helix-loop-helix factors such as Twist, ZEB1, ZEB2/SIP1, and TCF3/ E47/E12, transcriptionally repress E-cadherin the main constituent of adhesion junctions. Diverse upstream signals increase the expression of these E-cadherin repressors at the mRNA level. The most relevant factors are the transforming growth factor-beta (TGFβ)/bone morphogenic protein (BMP) family of cytokines, released from cancer-associated stromal cells such as fibroblasts that, differently from their normal counterpart, secret excess amounts of extracellular matrix (ECM), growth factors and chemokines [45]. The acquisition of EMT-like phenotype by cancer cells is reminiscent of *cancer stem-like cells*. Along these lines, a prominent role for ZEB1-mediated EMT has been identified [46]. The authors suggest that ZEB1 links EMT activation and stemness maintenance, by suppressing stemness-inhibiting microRNAs. On the other hand the silencing of ZEB1 not only induces EMT reversion, but also increases cellular sensitivity to therapeutic agents [47]. This is in line with the concept that the EMT program is linked to drug resistance and can be induced in cancer cells by stress conditions such as exposure to radiation or to anticancer agents [48, 45]. Shah and coauthors have reported that gemcitabine-resistant pancreatic cancer cell lines established by continuous exposure to the drug, can undergo EMT with increased expression of Snail and Twist [49], supporting the idea that therapeutic strategies to reverse EMT should be evaluated, considering the molecular factors inducing the EMT process.

These concepts may be important also in the context of molecularly targeted therapies, particularly those directed against EGFR. EGFR overexpression has been reported in pancreatic cancer [50, 51] and up to 60% of pancreatic surgical specimens overexpress EGFR [52]. Tzeng and colleagues reported that there are no EGFR mutations in pancreatic cancer [53], whereas alternative mechanisms, such as EGFR genomic gain,

aberrant ligand stimulation, receptor activation, absence of negative regulatory feedback molecules, and activation of downstream adaptor proteins like ERK1/2 and Akt support pancreatic tumor dependence on EGFR signaling. However, clinical results with both anti-EGFR mAbs and small-molecule TKI have been less than optimal (see Figure and Table 1). Mechanisms aside from mutation of the EGFR tyrosine kinase domain must therefore dictate drug sensitivity. *In vitro* and *in vivo* studies demonstrated that EMT status may be an indicator of sensitivity to EGFR inhibitors [54]: cell lines classified as sensitive expressed the canonical epithelial marker E-cadherin and displayed the classic cobblestone epithelial morphology and the tight cell–cell junctions of epithelial cells. Conversely, relatively insensitive cell lines lacked epithelial markers and expressed proteins characteristic of mesenchymal cells, including vimentin, fibronectin and ZEB-1 and exhibited a more fibroblastic, scattered morphology. Among the events leading to EMT, the disassembly of adherens junctions and the acquisition of a more motile and invasive phenotype through a significant reorganization of the actin cytoskeleton, play a crucial role. Human Mena (hMena) is a member of the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family, key actin cytoskeleton regulatory molecules controlling cell shape, movement, and actin organization at cadherin adhesion contacts. Experimental data suggest that hMena couples tyrosine kinase signalling to the actin cytoskeleton. Pino et al. showed that the expression of hMena, and particularly of its epithelial specific splice variant isoform hMena^{11a} [55], was restricted to the cancer cell lines that were E-cadherin positive and negative for the expression of vimentin and N-cadherin demonstrating that hMena^{11a} is a marker of an epithelial phenotype in pancreatic cancer cell lines [56]. Moreover, the expression of hMena/ hMena^{11a} is predictive of in vitro response to erlotinib, thus strongly supporting prospective studies to assess whether this molecular signature may be associated with an improved clinical response to EGFR targeted therapy in pancreatic cancer.

Novel molecular targets: Apoptotic pathways.

Programmed cell death or apoptosis is a critical process that maintains normal tissue homeostasis. Two crosstalking pathways regulate apoptosis and both ultimately trigger caspase activity: in the intrinsic pathway, mithocondria play a key role and the apoptotic process occurs for an imbalance between pro- and anti-apoptotic members of the Bcl-2 family of proteins; the extrinsic pathway is activated by the interaction between soluble or membrane-bound death-inducing ligands and death receptors of the tumor-necrosis factor receptor (TNFR) superfamily, including TNFR, FAS and TRAIL (TNF-related apoptosis inducing ligand) receptors on the cell surface [57].

Resistance towards apoptosis is a hallmark of pancreatic cancer [57]. In PanIN 1 and 2 lesions, no apoptotic cells could be detected, arguing for the contribution of anti-apoptotic mechanisms early in the carcinogenesis of PDAC [58]. Studies in pancreatic cancer have described multiple defects in apoptosis signaling at different levels of the pathway. Deregulated expression of apoptosis-regulating molecules, including members of the Bcl-2 family, such as Bcl-2, Bcl-XL, Bax, and Bak, is a common feature in PDAC. It has been shown that acquired resistance of pancreatic cancer cell lines to 5-FU and gemcitabine is associated with an alteration in the Bax/Bcl-xL ratio in favor of the activation of antiapoptotic genes [59]. Dong et al. demonstrated upregulation of Bcl-2 and decreased Bax expressions in micro-RNA-21 (miR-21) mimic-transfected cells. The increased expression of Bcl-2 was accompanied by less apoptosis, lower caspase-3 activity and decreased chemosensitivity to gemcitabine, compared with negative control cells. Opposite trends were found in the cells transfected with miR-21 inhibitor[60]. Furthermore expression of the proapoptotic Bax gene in tumor samples from patients with PDAC is a strong indicator of a longer survival and overexpression of Bax may sensitize pancreatic cancer cells to 5-FU and gemcitabine [61]. Bcl-xL also plays a role in protecting PDAC cells from FAS and TRAIL-mediated apoptosis and is constitutively overexpressed in pancreatic cancer cell lines highly resistant to Fas and TRAIL-mediated apoptosis [59]. While overexpression of Bcl-xL in cell lines with endogenously low levels induces complete suppression of apoptosis, inhibition of Bcl-xL function by either antisense oligonucleotides or Bax overexpression results in sensitization of cells with high levels of Bcl-xL, such as Panc-1 or Panc-TuI, resulting in the inhibition of pancreatic cancer cell growth, induction of apoptosis, and increased sensitivity to chemotherapeutic agents

like gemcitabine [59]. BNIP3 is a hypoxia-inducible mithocondrial member, belonging to the BH3 subfamily of Bcl2 family proteins, which antagonizes the activity of prosurvival proteins such as Bcl-2 and Bcl-xL [62]. Under physiologic conditions BNIP3 is usually undetectable in most tissue while it has been reported in hypoxic regions. Despite the characteristic hypoxic milieu of the pancreatic cancer (see below), several studies show that BNIP3 expression levels in PDAC cell lines and tissue samples are low, as compared to the normal pancreas, possibly due to hypermethylation of the BNIP3 promoter [62]. Recently Erkan et al. demonstrated alterations of BNIP3 expression during transformation, although the major loss of the protein occurs in later stages of PDAC development and correlates with poorer median survival [61]. In addition, downregulation of BNIP3 results in increased resistance to 5-fluorouracil and gemcitabine [63], although the latter point is more controversial [62]; regardless, BNIP3 expression may have an impact on prediction of prognosis of patients with pancreatic cancer [62].

Human pancreatic cancer tumors or cell lines exhibit heterogeneous responses to TRAIL, and some of them are intrinsically resistant to TRAIL-induced apoptosis [64], such as human pancreatic cancer cell lines harboring codon 12 mutations of the K-Ras gene [65, 66]. Recent experimental data showed that the histone deacetylase inhibitor LBH589 is able to promote ubiquitin/proteasome-mediated degradation of the TRAIL inhibitor c-FLIP, thereby sensitizing PDAC cells to TRAIL-induced apoptosis [67, 68, 69].

Similarly, it has been reported that treatment with cycloheximide, a compound able to suppress FLIP and TRAIL expression, may enhance and restore apoptosis in TRAIL-resistant cells [70]. Mori et al. reestablished TRAIL-induced apoptosis in TRAIL-resistant cells by exposure to FLIP antisense; moreover, the effect of TRAIL was enhanced by the combination of FLIP antisense and embelin, a small inhibitor of X-linked inhibitor of apoptosis protein (XIAP) [71]. Other data support the increase of the apoptosis-sensitization of TRAIL-resistant pancreatic carcinoma cells by down-regulation of c-FLIP and XIAP[72, 73, 74, 75]. TRAIL has a great potential for clinical application and agonistic TRAIL receptors (TRAIL-R1/R2) are being investigated as therapeutic targets. The agonistic antibody to the proapoptotic TRAIL receptor TRAIL-R, Mapatumumab, has been showed to reduce viability in pancreatic carcinoma cells and to cooperate with XIAP inhibitor to trigger caspase activation in *in vivo* tumor models [76], and is currently being tested in clinical trials.

The therapeutic potential of XIAP inhibition in PDAC, is highlighted by evidence that small-molecule XIAP inhibitors synergize with TRAIL to induce apoptosis and inhibit survival of PDAC cells *in vitro* and act in concert with TRAIL to suppress the growth of established pancreatic cancer *in vivo* [77]. Karikari et al. showed that XAntag, a functional inhibitor able to disrupt the interaction of XIAP with caspases, is capable of activating downstream caspases in pancreatic cancer cells and of inhibiting pancreatic growth *in vivo*. XAntag also showed synergy when combined with proapoptotic ligands (TRAIL) and sensitized pancreatic cancer cells to common pancreatic cancer therapy, such as radiation and gemcitabine [78]. In addition to XIAP, data report that two other members of the inhibitors of apoptosis (IAP) protein family, cIAP2 and survivin are overexpressed during carcinogenesis of PDAC. Early overexpression of cIAP2 was demonstrated in 30% of low-grade PanIN lesions, 50% of high-grade PanIN lesions and 85% of PDAC [57]. Survivin is also often overexpressed in pancreatic cancer, where it participates in the development and progression of the disease and correlates with poor prognosis [79]. Guan et al. investigated the effects of a small interfering RNA (siRNA) directed against survivin, showing that it inhibits surviving expression in pancreatic cancer cell lines, induces apoptosis, and enhances radiosensitivity [80].

Novel molecular targets: HIF and angiogenesis.

The hypoxia inducible factor (HIF) family of transcription factors plays a crucial role in response to hypoxia-specific stress and is involved in resistance to oxidative stress, protecting the cancer cell against cytotoxic therapy and inversely correlating with sensitivity to ionizing radiation. HIF-1 is a heterodimer composed of α and β subunits. While HIF-1β is constitutively expressed, HIF-1α is regulated in an oxygen-dependent manner at the post-translational level and is undetectable under normoxic conditions. Hypoxia stabilizes HIF-1α, which then translocates to the nucleus, dimerizes with HIF-1β and activates the transcription of hypoxia-regulated genes [81]. Several studies showed that HIF-1α positive cells are mostly localized at a tumor's infiltrating margin, around the periphery of necrotic areas, and surround regions of high

microvessel density . HIF-1 overexpression may represent an early event in carcinogenesis and seems to play an important role also in cancer growth and metastasis [82].

The overexpression of HIF-1α has been demonstrated in multiple types of human cancer, as well as in their regional and distant metastases, as a result of adaptation of tumor cells to hypoxia. HIF-1 overexpression, in turn, is related to the up-regulation of proangiogenic cytokines such as VEGF and platelet-derived growth factor (PDGF), which confer radiation resistance to endothelial cells as well as increase the proliferation of tumor blood vessels [83, 84]. Furthermore, HIF- 1 transactivates various hypoxia-responsive genes, which confer malignant properties to tumors such as apoptosis resistance, enhanced tumor growth, invasion and metastasis [85]. Overexpression of HIF-1α and its role in angiogenesis and progression is associated with unfavorable prognosis and poor overall and disease free survival in several cancers [83], including PDAC, in which HIF-1 activation in response to low oxygen levels has been demonstrated both *in vitro* and *in vivo* [82]. Recently, Sun et al. and Tao et al. showed that HIF-1α expression not only has a strong impact on the prognosis of PDAC patients, but is also correlated with decreased apoptotic index, increased intratumoral microvessel density and VEGF expression [86, 87]. It has be further investigated to what extent HIF-1α and PDGF-A are coexpressed in pancreatic cancer [88]. Recent data show a correlation between hypoxic environment, induced HIF-1α expression and pancreatic cancer cells resistance to gemcitabine [89]; furthermore HIF-1α overexpression seems promote EMT and facilitate invasiveness of pancreatic cancer cells [90, 91].

Targeting HIF-1 is a possible therapeutic strategy. Inhibition of HIF-1 by PX-478 in cancer cell lines is able not only to reduce HIF-1 protein levels and signaling but also to provide direct radiosensitization of hypoxic cancer cells and improvement in direct tumor cell killing [92]. Chen et al. showed that siRNA may inhibit HIF-1 and VEGF expression inducing apoptosis of pancreatic cancer cells through NF-κB-independent or -dependent pathways under hypoxic conditions and might inhibit the growth of nude mice xenografts and their microvessel density (MVD) [93].

Novel molecular targets: Developmental pathways.

The hedgehog pathway

The hedgehog (Hh) pathway plays an important role during embryonic development, although it is usually inactive in mature organs. Hedgehog belongs to a family of secreted signaling proteins comprised of sonic, indian, and desert hedgehog (SHh, IHh, and DHh, respectively). It functions by binding to a transmembrane protein called patched (Ptch), a tumor suppressor protein that negatively regulates the Hh pathway. When Hh ligands bind to their receptors, Ptch releases its inhibitory protein smoothened (Smo), which activates the downstream effectors and determines the nuclear localization of the Gli transcriptional regulators with upregulation of target genes [94].

Aberrant activation of the Hh pathway by loss of Ptch, mutation in Smo and overexpression of Gli and Hh proteins, have been linked to tumorigenesis in several cancers. Pancreatic cancer, in particular, shows an aberrant expression of SHh secreted in an autocrine/paracrine manner [10]. SHh pathway plays a key role in the growth of pancreatic cancer cells and seems to be implicated in initiation and maintenance of pancreatic ductal cancers. SHh is aberrantly expressed in the precursor lesions of invasive adenocarcinoma, such as PanIN and intraductal papillary mucinous neoplasia, and its levels increase progressively as the lesions progress to more advanced stages [95]. Some of the genes related to the Hh pathway are tumor suppressors or oncogenes, several Hh-target genes are overexpressed and there is a considerable degree of cross-talk between Hh and other oncogenic/oncosuppressor pathways, including the MAPK/ERK, PI3K/Akt, Wnt, and TGFβ/BMP pathways [96]. For example, RAS and Hh cooperate during carcinogenesis, initiation, and maintenance in the pancreas and other organs. Lauth et al. found that mutant RAS induces and enhances SHh expression, favoring paracrine Hh signaling, whereas antagonizes autocrine Hh signal [97, 98]. SHh has been also linked to cell survival and apoptosis. Morton at al. showed that SHh expression protects pancreatic cancers form caspase 8- and caspase 3-dependent apoptosis, in part through stabilization of the anti-apoptotic proteins Bcl-2 and Bcl-xL [97]. Other data have shown that SHh can

rescue cells from apoptosis mediated by the FAS death receptor, allowing protection from tumor-reactive immune cells [97] and that Hh signaling plays a role in pancreatic cancer stem cells, driving their self-renewal ability [99, 100]. Finally several data have shown that a ligand dependent activation of SHh occurs in the tumor stromal microenvironment where it is involved in myofibroblast differentiation and induces desmoplasia. Moreover, a hypoxia-driven upregulation SHh, Smo and Gli transcription and a SHh paracrine loop, through induction of stroma-derived growth factors, have been demonstrated [101, 102, 103, 104, 105].

All these evidences raised interest in targeting this pathway for the treatment of cancer. Several small molecule inhibitors are being evaluated in clinical trials, either as a therapy directed against tumor cells, or as a strategy against non-malignant stromal cells that support the growth of the tumor [99, 106]. Cyclopamine, a natural steroid alkaloid antagonist of Smo, has been shown to inhibit the growth of murine cancer cell lines, downregulate the HH target genes Gli1 and Ptch, selectively deplete the ALDH-positive subpopulation of PDAC stem cells, impair invasive capacity, prevent metastasis, and significantly prolong survival in murine orthotopic xenograft models or transgenic mouse models of human pancreatic cancer [107, 108]. It has also been shown that tumor vasculature is a target of Hh blockade. Cyclopamine is able to attenuate Hh signaling in the stroma, determining regression of the tumor vasculature and attenuating the recruitment of bone marrow (BM)-derived cells [96]. Olive et al. showed that IPI-926, another small-molecule inhibitor of SMO reduce the dense stromal reaction that characterizes pancreatic cancers and transiently increases tumor neovascularization, thereby facilitating drug delivery and increasing the survival of mice in an otherwise gemcitabine-resistant mouse model [106, 109]. Furthermore GDC-0449 (Vismodegib), a Smo inhibitor with a high degree of selectivity for SHH-Gli signaling has been showed to induce significant cell death in three pancreatic cancer cell lines (AsPC-1, PANC-1 and MIA PaCa-2) and pancreatic cancer stem cells (CSCs) by decreasing SHh signaling components (Gli1, Gli2, Patched-1, Patched-2, SHH and Smo) expression, Gli-DNA binding and Gli-luciferase reporter activities. In addition, GDC-0449 increased the expression of TRAIL-R1/DR4 and TRAIL-R2/DR5, decreased Bcl-2 expression, and induced caspase-3 activity [110].

The Notch pathway

The developmental signaling pathway Notch plays a fundamental role in cell fate decisions during embryogenesis and is important for the process of apoptosis, differentiation and invasion [100]. During pancreatic development, Notch signaling maintains pancreatic epithelial cells in a progenitor state, avoiding the premature differentiation of pancreatic progenitors [111, 112]. In adult tissues, Notch prevents cell terminal differentiation and maintains the subpopulation of undifferentiated stem/progenitor cells [113]. In the adult pancreas, under normal conditions, Notch is relatively inactive, while it plays an important role in pancreatic homeostasis and actively participates in the regeneration process following pancreas injury, potentially providing an important link between chronic pancreatic inflammation and pancreatic carcinogenesis [114]. The Notch pathway includes 4 different single-pass transmembrane receptors. The Notch signaling can be initiated by the binding of one of five distinct ligands (JAGGED-1 and 2 and Delta-like (DLL)-1, -3, and -4) to the receptors [111]. The receptor-ligand interaction induces the proteolytic intramembrane cleavage of the receptor by γ-secretase and the release of an active intracellular domain (Notch intracellular domain, NICD), which then translocates to the nucleus. Here, the NICD induces the expression of a series of target genes, including the transcriptional repressors family of the hairy enhancer of split (Hes) and Hes-related family of genes [112].

When aberrantly regulated, Notch can contribute to cell transformation *in vitro* and to the development of several human cancers [10]. In pancreatic cancer, the Notch pathway plays a role in initiation, progression, and maintenance [112, 115, 116]. Activation of the Notch pathway has been observed in metaplastic ductal epithelium, in early, non-invasive stages of disease (such as PanIN), and in PDAC tissue in human and mouse models [112]. Overexpression of NICD has been shown to be sufficient to promote acinar-ductal metaplasia in primary acinar cell culture and cooperates with other signaling pathways to initiate pancreatic carcinogenesis [112]. Moreover, Notch signaling seems to play a role in the acquisition of EMT and cancer stem-like cell phenotype and its activation has been shown to be associated with a

chemoresistance phenotype of PDAC cells [117, 118]. No somatic point mutations either in Notch genes or in genes encoding their ligands have been identified. Indeed mutational activation of Notch pathway in pancreatic cancer is rare. Rather, Notch activation in pancreatic cancer seems to be ligand driven. Mullendore et al showed an endogenous overexpression of Notch ligands, especially of JAGGED2 and DLL4, in pancreatic cancer cell lines, which correlated with induction of the transcriptional target gene Hes [111]. Notch-1 has been reported to cross-talk with other survival/embryologic pathways. Several data demonstrate interactions of Notch with RAS, in both development and tumorigenesis. Activated KRAS and Notch signals cooperate to transform cells and to initiate pancreatic carcinogenesis and Notch activity accelerates the formation of oncogenic KRAS-induced PanIN lesions [10, 112, 119]. Notch-1 has been reported to crosstalk with another major cell growth and apoptotic regulatory pathway: nuclear factor-κB (NF-κB). Specifically, Notch-1 has been reported to strongly induce NF-κB2 promoter activity and expression of several NF-κB subunits [117].

Notch is a potential therapeutic target for pancreatic cancer and blocking its signal transduction, by small-molecule inhibitors, showed antineoplastic effects *in vitro* and *in vivo*. Wang et al showed that tumor maintenance in pancreatic cancer is mostly due to NOTCH1 activity and that its inhibition might affect cell growth and survival, prevent invasion by inhibiting NF-κB, VEGF and MMP [120]. Genistein, a natural isoflavonoid found in soybean products, and curcumin, a phenolic compound from the plant *Curcuma longa*, have been shown to down-regulate the transcription and translation of Notch-1 and Hes-1 and to inactivate NF-κB. In addition, these drugs induced G0-G1 phase cell cycle arrest, with reduced levels of cyclin D1, and induced apoptosis by decreasing Bcl-2 and Bcl-xL protein expression [111, 121]. Notch down-regulation by siRNA also reduces VEGF expression and decreases MMP-9, resulting in the inhibition of invasion and metastasis [122]. A different therapeutic strategy is the prevention of Notch activation by γ-secretase inhibitors, which in several reports is correlated to reduced proliferation, increased apoptosis, decreased anchorage-independent growth and invasion properties in pancreatic cell cultures [111,123]. γ-secretase inhibition is able to prevent acinar-to-ductal metaplasia in vitro, suppress the incidence of PanIN,

and block the progression of PanIN to PDAC in mouse models, but does not seem to exert a strong influence during more advanced stages of PDAC progression [112].

Animal models of PDAC.

The most commonly used animal models are cell line-derived tumor xenografts in immunodeficient mice [124]. However, this approach is not able to recapitulate some important features of the original disease, such as interaction with the immune system, stromal and endothelial cells, and the normal epithelial environment [125, 126], making the predictive value of therapeutic experiments conducted in these models at least questionable, particularly in PDAC where the interaction with 'normal' stroma is of paramount importance [6, 127].

As an alternative to cell line-based in vivo models, Hidalgo and coll. have developed individual patient-derived PDAC xenotransplantation models, with the advantage of retaining the genetic and epigenetic features, as well as the tumor heterogeneity, of the original disease [128, 129]. Although this approach has proven feasible and potentially useful for preclinical/early clinical drug development [130], it still lacks substantially with regard to the role of tumor-host interactions. This issue is theoretically overcome by the genetically engineered mouse models [131, 132], which allow modeling the interplay between neoplastic cells and the surrounding environment, providing an in situ tumor development in an immune-competent model [126].

With specific regard to PDAC, several transgenic models have been developed [133]. Among these, KPC mice are genetically engineered models, which conditionally express endogenous mutant K-RAS and p53 alleles in pancreatic cells, leading to the development of pancreatic tumors [126, 134]. PDAC in KPC mice recapitulates patho-physiological and molecular features of human PDAC, including the development of tumors with abundant stroma and collagen deposition, the common occurrence of metastasis to relevant sites, cachexia and activation of biochemical pathways and genomic instability [126]. Recent reports show that K-RAS activation in mouse pancreas generates pancreatic intraepithelial neoplasia (PanIN), with a low

frequency of progression to invasive and metastatic adenocarcinoma, thus providing a pre-invasive pancreatic cancer model that offers interesting opportunities for chemoprevention and early detection research. Indeed, serum samples from such mice are able to show specific changes in serum protein profiles that could represent specific markers of pancreatic disease (see also next chapter). Additional studies have demonstrated the specific role for signaling pathways, such as Hedgehog, EGF and Notch, in regulating normal pancreatic development in the mouse and in both PanIN and invasive pancreatic cancer, improving the understanding of human pancreatic cancer biology [130] and opening new perspectives for targeted treatment of such a deadly malignancy.

Proteomics as a tool for the identification of new therapeutic targets.

The search for biomarkers by analyzing gene overexpression or protein elevation in pancreatic juice or in sera has led to the identification of several tumor targets, including mesothelin, macrophage inhibitory cytokine-1 (MIC-1) and osteopontin [135]. Various technologies have been recently employed for the identification of candidate PDAC biomarkers using large-scale analysis of antigen expression (based on either RNA or protein levels). In particular, proteomic technologies have been used to detect antigens, which can be resolved by two-dimensional gel electrophoresis (2- DE) and identified using Mass Spectrometry and which elicit a humoral response in the sera of PDAC patients [136]. Variants of this approach for protein separation, selection and characterization have been described under different names in the literature, such as SERPA [136] PROTEOMEX [137], or SPEAR [138]. Lists of candidate PDAC-specific proteins have been generated on the basis of their elevated expression at the RNA level or of large-scale proteomic analysis of serum and/or pancreatic tissue samples (recently reviewed in [139]).

We have recently screened the reactivity of IgG in PDAC sera against proteins from PDAC cell lines (CF-PAC-1 and MIAPACA-2) resolved by 2-DE. The reactivity of IgG from PDAC sera was compared to that of IgG from sera of chronic pancreatitis (CP), non-PDAC tumor patients and healthy subjects (HS). Approximately 10 to

60% of PDAC patient sera contain IgG against two functional kinds of proteins: metabolic enzymes, namely α-enolase (ENOA), triosephosphateisomerase 1 (TPIS), retinal dehydrogenase 1 (AL1A1), glucose-6-phosphate 1-dehydrogenase (G6PD), elongation Factor Tu (EFTU), and isocitrate dehydrogenase (IDHC) and cytoskeletal proteins, namely keratin 10 (K1C10), cofilin-1 (COF1) and transgelin (TAGL) [140, 141, 142]. Antibodies to these proteins were detected at very low frequency in sera from healthy subjects, chronic pancreatis (CP), autoimmune and non-PDAC tumor patients, suggesting that the antibody response to them is characteristic of PDAC, although antibodies to some of these proteins are produced in other types of cancer (such as antibodies to TPIS and K1C10 in sera from lung and kidney cancer patients) [143, 144]. The specificity of the antibody response for pancreatic cancer was further underlined by the observation that, with the exception of AL1A1, all of the proteins recognized by antibodies in PDAC sera were upregulated in PDAC biopsies [140, 141], in agreement with data reporting TPIS, K1C10, EFTU, IDCH and COF1 overexpression in PDAC [144]. An interesting point that comes from these studies is that PDAC is characterized by an antibody response to the cytoskeletal proteins K1C10 and COF1. In particular, the most frequent antibody response of PDAC patients is directed against COF1, an actin depolymerizing protein involved in invadopodium formation and required for tumor cells directionality in response to chemotactic or growth-factor stimulation [145]. K1C10 is also involved in the formation of the intermediate filament cytoskeleton of all epithelial cells [146]. Although autoantibodies to cytoskeletal proteins have been found in cancer [147] autoantibodies to COF1 in PDAC have not been described so far. Of interest, COF1 might be functionally associated with TPIS to feed glycolic fuel to Na, K ATPase [148]. As the most frequent antibody response in PDAC patients is directed to COF1 and TPIS2, our data support the hypothesis that these two proteins play a role in the biology of pancreatic cancer.

Interestingly, from a clinical perspective, another antigen highly recognized by PDAC patients [141, 142], ENOA, is also expressed on the surface of many cell types including PDAC where it acts as a plasminogen receptor leading to extracellular matrix degradation and cell invasion [149]. As cancer progression is often associated with the ability to escape immune responses [150], the induction of autoantibody production may reflect a more efficient immune response in PDAC patients. This hypothesis is supported by the

observation that the peripheral blood of PDAC patients with autoantibodies against ENOA contains ENOA-specific $CD4^+$ and $CD8^+$ T cells [141]. In this context, the presence of autoantibodies to ENOA in PDAC patients might directly reflect the activation of anti-ENOA $CD4^+$ T helper cells. The expression of ENOA is upregulated in PDAC at both the mRNA and protein levels [141, 142, 151, 152]. 2-DE and WB analysis with an anti-ENOA monoclonal antibody demonstrated that ENOA consists of six different isoforms (ENOA1,2,3,4,5,6) with a similar 47 kDa molecular weight and pI ranging from 6.6 to 8 [142, 153]. A further characterization of the each ENOA spots from PDAC and normal pancreatic duct cells by reversed-phase liquid chromatography nanospray tandem mass spectrometry (LC-MS/MS) analysis identified multiple novel post-translational modifications of ENOA, such as phosphorylation, acetylation, and methylation [153]. By this approach, we identified for the first time a unique phosphosphoserine residue in the position 419 of ENOA. By 2-DE WB we have found that phosphorylated ENOA1,2 isoforms are preferentially expressed in PDAC tissues and tumor cell lines and, to a lesser extent, in normal pancreatic tissues [142]. Most importantly, we have documented that ENOA1,2 isoforms induce an *in vivo* humoral response in a substantial proportion of PDAC patients (62%). We provided evidence that such reactivity is directed against the phosphorylated residue and is specifically associated to PDAC, in that it is only sporadically observed in patients with inflammatory pancreatic diseases (CP), autoimmunity, and patients with non-PDAC malignancies [142]. Of clinical relevance, ENOA1,2 autoantibodies are significantly more frequent in patients with normal CA19.9 levels, potentially complementing the performance of the most widespread serological test currently employed in the diagnostic workup of both cystic and solid pancreatic masses. Indeed, we have demonstrated that the combination of CA19.9 and anti-ENOA autoantibodies yields a diagnostic accuracy of approximately 95%, not only in advanced PDAC patients, but also in patients who may be amenable to surgery with curative intent. Finally, anti-ENOA1,2^+ patients exhibit a more favorable clinical course with a significantly lower proportion of disease progression and a consequently longer PFS upon gemcitabine treatment [142]. This is clinically relevant in a disease, such as advanced PDAC, where only performance status and disease stage have proved to reliably predict clinical outcome [154].

Further investigations are needed to validate PDAC sera reactivity against phosphorylated ENOA1,2 and other identified tumor associated antigens in a large-scale study, as well as to fully evaluate their clinical usefulness.

Summary and conclusions.

Despite the disappointing clinical results obtained so far with molecularly targeted agents in advanced PDAC, it is an exciting time for pancreatic cancer basic and translational research. Novel approaches, such as molecular epidemiology and proteomics, are beginning to shed light on novel mechanisms underlying pancreatic cancer initiation and progression and are likely to yield novel therapeutic targets to be tested in the clinical setting. Moreover, the availability of recently developed, clinically relevant animal models of PDAC [155, 109] should now enable a more extensive preclinical testing of novel therapeutic strategies, thereby allowing a more efficient selection of promising approaches for clinical testing and ultimately increasing the rate of success of clinical trials testing novel agents for pancreatic cancer therapy. Exciting developments in the understanding of the molecular and genetic bases of pancreatic cancer should therefore raise renewed hope for all of our patients affected by such a devastating disease.

Table 1. Sensitivity analysis of randomized trials comparing TA-G vs G, according to the pathway targeted by TA.

Pathway	Pts[a] (RCT)	Outcome	HR/RR (95% CI)	*p*-value	Heterogeneity (*p*-value)
EGFR[14, 156]	1,304 (2)	OS[b]	0.93 (0.73-1-20)	0.61	0.03
		PFS[c]	0.97 (0.80-1.16)	0.75	0.81
		ORR[d]	0.90 (0.65-1.24)	0.53	0.40
FT[157]	688 (1)	OS	1.03 (0.86-1.23)	0.74	1.00
		PFS	0.98 (0.78-1.22)	0.86	1.00
		ORR	0.72 (1.41-1.26)	0.25	1.00
MMP[19]	239 (1)	OS	0.99 (0.75-1.29)	0.94	1.00
		PFS	1.01 (0.72-1.42)	0.93	1.00
		ORR	0.68 (0.30-1.64)	0.36	1.00
VEGF[158, 18]	1,232 (2)	OS	1.03 (0.89-1.18)	0.67	0.85
		PFS	1.01 (0.90-1.13)	0.97	0.82
		ORR	0.86 (0.66-1.11)	0.26	0.056

[a]Pts: number of patients; RCT: number of randomized clinical trials; HR: hazard radio (for survival outcomes); RR: relative risk (for overall response rate); CI: confidence intervals; EGFR: epidermal growth factor receptor; MMP: matrix metallo-proteases; FT: farnesyl transferase; VEGF: vascular endothelial growth factor; OS: overall survival; PFS: progression free survival; ORR: overall response rate.

[b]Interaction p for OS =0.82

[c]Interaction for PFS =0.95

[d]Interaction p for ORR =0.33

Table 2. Inherited cancer syndromes at increased lifetime risk of developing PDAC.

Syndrome	Gene (Chromosome)	PDAC RR	Frequency in sporadic cases	Cancers common to the syndrome
Peutz-Jeghers syndrome	*STK11/LKB1* (19p13.3)	132	4%	GI (esophagus, stomach, pancreas, small and large bowel), endometrium, ovary testes, lung, breast
Hereditary pancreatitis	*PRSS1* (7q35)	≥ 20	n.a.	Pancreas
Familial atypical multiple mole melanoma (FAMMM)	*P16INK4a/MTS1* (9p21)	13-22	98%	Benign nevi, malignant melanoma, endometrial, pancreas, lung, breast
Hereditary breast/ovarian cancer (HBOC)	*BRCA1* (17q21-24) *BRCA2* (13q12-13)	2.3-6 3-10	n.a. ~ 7%	Breast, ovary, pancreas, stomach, colon fallopian tube, gallbladder, bile duct, malignant melanoma
Hereditary nonpolyposis colorectal cancer (HNPCC)	*MSH2* (2p22-21) *MLH1* (3p21.3)	n.a.	4-11%	Colorectal, pancreas, biliary tract, papilla of Vater
Familial adenomatous polyposis (FAP)	*APC* (5q21)	~ 5	40%	Colorectal adenomas, colorectal cancer, pancreas

Figure legends.

Figure 1. Forest plot analysis of OS results in randomized trials of TA-G vs G.

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