REVIEW

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The Molecular Targets for the Diagnosis and Treatment of Pancreatic Cancer

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Pancreatic cancer is considered an aggressive malignancy that responds poorly to current treatments and therefore has a dismal survival rate. This disease is usually not diagnosed until a late stage, at which point palliative chemotherapy with the purine analogue gemcitabine and/or a fluoropyrimidine or a platinum agent is the standard approach. There are some new data on the molecular and genetic changes that take place in pancreatic cancer, which may facilitate the accuracy of diagnosis and efficacy of treatments. However, translational efforts in clinical practice have increased clinicians' options with a targeted agent, erlotinib, in combination with the standard gemcitabine chemotherapy. Many other novel drugs currently being tested in the field of pharmaco-oncology target various altered biological pathways and molecules. Nevertheless, the lack of clinically significant improvements in treatments is rendering efforts to develop methods of early diagnosis both more urgent and promising. The aim of this review was to summarize the molecular basis of pancreatic carcinogenesis and the latest developments in diagnosis by molecular means, focusing on the results of clinical research into targeted and personalized treatments. **(Gut Liver 2010;4:433-449)**

Key Words: Pancreatic ductal carcinoma; Molecular targets; Pharmacogenetics; Novel agents

INTRODUCTION

Pancreatic cancer (PC) is the 4th commonest cause of cancer related deaths according to statistics for 2008 by the American Cancer Society. The mortality rate of pancreatic cancer is very high (99%) and the 5-year survival rate for all stages equal or less to 5%. The incidence of this lethal disease is fortunately much lower, representing only 2% of all cancers (10th commonest cause) in United States and rather the same in the rest of western world.

There are few risk factors that have been identified in the sporadic form of pancreatic cancer which accounts for the 90% of all cases (genetic syndromes are accountable for the rest 10%). Such risk factors are cigarette smoking, age >55 years, obesity, lack of exercise, male gender and possibly but less certainly chronic pancreatitis and diabetes type II.[1] The fact that most of the above factors have showed an increasing tendency during the last decades may explain why the mortality rate is not slowing down despite improvements in treatment.

The gold standard treatment for early stage pancreatic cancer is radical surgery (Whipple's operation) which is actually the sole curative option in this aggressive tumor. Chemotherapy can be used as adjuvant to surgery or in advanced stage pancreatic cancer where, in a small group of patients, it offers real benefit in terms of survival and quality of life. In addition, radiotherapy may offer in selected cases local control in advanced nonmetastatic disease when surgery is either not feasible or incomplete.

Due to poor results of the conventional treatments, a labor effort in translational science is taking place over the last decade aiming to an earlier diagnosis and a more effective treatment. Below, we will focus on the aberrant

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biological pathways involved in the pathogenesis of pancreatic cancer and the deranged molecules or genes that are attracting diagnostic or therapeutic interest. Finally, we will present the current status of novel treatments produced in drug development units which may allow applying a more rational patient's management.

GENETIC AND MOLECULAR BACKGROUND OF PANCREATIC CANCER

There are many different histological subtypes of pancreatic cancer, with variable natural history, management and outcome. Pancreatic ductal adenocarcinoma (PDAC) is the commonest subtype followed by cystic neoplasms (serous cystadenocarcinoma, intraductal papillary mucinous neoplasm-IPMN), neuroendocrine tumors, sarcoma, acinar cell carcinoma and lymphoma. Though there is evidence that PDAC may also develop on the background of mucinous neoplasms (IPMN or mucinous cystic), we will not deal with the molecular aspects of those rather rare cases in this review. In the majority of published works, the term pancreatic cancer refers exclusively to PDAC.

The carcinogenesis of pancreatic neoplasms entails transformation of a normal cell to a benign or premalignant cell, as those seen in pancreatic intraepithelial neoplasia (PanIN). Various genetic mutations, progressive nuclear alterations, such as increasing atypia and loss of polarity, as well as morphological cellular changes do occur and mount up during the malignant process from the early PanIN1to the more advanced PanIN3 or carcinoma *in situ* and finally pancreatic cancer.[2,3]

Therefore, the observed genetic mutations in this disease involve the oncogenes *KRAS* in the majority of cases (74-100%), *HER-2/neu* (in about 65%), *notch1*, *Akt-2* and

Table 1. Most Common Molecular Alterations in Pancreatic Cancer and Applicable Targeted Agents

Genes	Role	Frequency of alteration	Selective targeted agents	Clinical significance
KRAS	Oncogene	74-100%	Farnesyl transferase inhibitors (FTIs) Tipirfanib, lorafarnib	• Mutation at codon 12 may be a negative prognostic factor • FTIs not active in pancreatic cancer (PC)
HER2/neu	Oncogene	16-65%	Trastuzumab, lapatinib	No therapeutic benefit
HER3	Oncogene		Lapatinib, erlotinib	Might be associated with response to erlotinib
Akt2	Oncogene	10-72%	Silencing with RNA interference (RNAi) evaluated	
Notch-1	Oncogene	50-90%	Silencing with RNAi and inhibition by curcumin, genistein evaluated	Overexpression, not mutation
COX-2	Oncogene	40-50%	Celecoxib, apricoxib	Association with poor outcome and advanced stage
p16INK4a	Oncogene	27-96%	No selective inhibitor available	Confounding data regarding its prognostic value
p53	Tumor suppressor	43-76%	No selective inhibitor available	Confounding data regarding its prognostic value Probably predictive of poor response to Rx
DPC4	Tumor suppressor	50%	No selective inhibitor available	Controversial prognostic value
BRCA2	Tumor suppressor	6-17%	No selective inhibitor available	BRCA 1/2 genes involved in DNA repair BRCA2 implicated in the familial PC Benefit from PARP1 inhibitors in breast/ovarian Ca
FHIT	Tumor suppressor	70%	No selective inhibitor available	Unknown
EGF-R	Growth factor & receptor	25-65%	Cetuximab Erlotinib (Tyrosine kinase inhibitor, TKI) Gefitinib (TKI)	Not established prognostic or predictive role yet Cetuximab not active Erlotinib (and possibly gefitinib) active when combined with gemcitabine
VEGF-R	Growth factor & receptor	Up to 90%	Bevacizumab Aflibercept (VEGF trap) Vatalanib (TKI) Vandetanib (small molecule)	No therapeutic benefit yet Studies still in progress
MMPs	Matrix proteases	?	Marimastat Tanomastat Ro 28-2653	No therapeutic benefit yet
mTOR	Protein kinase	?	Temsirolimus Everolimus	No therapeutic benefit yet

COX-2, and the tumor suppressor genes *p16INK4a* (in up to 98%), *p53* (43 to 76%), *DPC4* (about 50%), *FHIT* (found in 70% of cases) and *BRCA2* in familial cases.[4-10]

Apart from single genetic changes there are specific chromosomal abnormalities involved in pancreatic carcinogenesis. Thus, we may see allelic loss mainly in chromosomes 17p (95%), 18q (88%), 9p (76%), 12q (67%) and less often in 1p, 6p, 6q, 8p, 10p, 10q, 12p, 21q, and 22q (from 50% to 60%). There are also cases where chromosomal additions do happen, such as in chromosomes 7 and 20.[11] What might happen in reality is a mixture of chromosomal and genetic changes as many tumor suppressor genes are positioned in the aforementioned locations for example *p53* at chromosome 17p, *DPC4* gene at chromosome 18q and *p16INK4a* (*MTS1*) gene at chromosome 9p.

1. Altered genes and clinical significance (Table 1)

1) *KRAS*

The Kirsten Rat sarcoma virus proto-oncogene (*KRAS*) is found in chromosome 12p at the position 12.1. The significance of RAS pathway in signal transduction from the cell surface receptors to the nucleus, affecting the production and regulation of other key proteins has been established in numerous published works. The main action of the three proto-oncogenes of the RAS family (*H-RAS*, *K-RAS*, and *N-RAS*), which are located in the inner plasma membrane, is the binding of GDP and GTP. RAS proteins possess and confer intrinsic GTPase activity which cleaves the GTP to GDP and leaves it in a "switch off" position. *KRAS* protein is active and transmits signals by binding to GTP (turn on), but it is inactive (turn off) when GTP is converted to GDP.

KRAS mutations are associated with inactivity of GTPase which subsequently leaves GTP at the "switch on" position. Increasing role of *KRAS* mutations has been recognized in many gastrointestinal tumors, mainly in colorectal adenocarcinomas. In pancreatic adenocarcinoma, the vast majority of tumors harbor *KRAS* mutations (from 74% up to 100% in various series).[12-16] The most frequent mutations observed are those in codon 12 followed by point mutations in codons 13 and 67.[14] The data about the prognostic and predictive significance of the above mutations of *KRAS* is rather limited and conflicting.[5,17] The high frequency of *KRAS* mutations in PC may in part explain the lack of response to epidermal growth factor receptor (EGFR) inhibitors, similarly to colorectal cancer patients.[18,19]

2) *p16/INK4*

Gene *p16* is a tumor suppressor gene, located in chromosome 9*p21*. This gene is also named as *INK4a*, *CDKN2* or *MTS1-multiple tumor suppressor 1*. Gene *p16* encodes for a protein (p16INK4a) which inhibits the interaction of cyclin D with the kinases CDK4 and CDK6 and thus inhibits cell cycle progression at the G1→S step. The cyclin D-CDK4 complex phosphorylates the retinoblastoma protein (Rb1), preventing thus the formation of the E2F-Rb1 complex and leaving E2F available to act as a transcription factor facilitating cell cycle progression. In pancreatic cancer cells inactivation of *p16INK4a* results in uncontrolled cell cycle progression due to absence of inhibition of the cyclin D-CDK4 complex.

In PC, inactivation of *p16* is caused by various means such as point mutation, hypermethylation or homozygous deletion of the gene, and is observed in the majority of these patients according to various published works.[16,20,21]

The prognostic significance of p16 is not established as there are conflicting data and therefore more evidence is needed before any clinical application.[22-24]

3) *p53*

This is the most known and studied tumor suppressor gene as it is frequently mutated in various neoplasms. In normal conditions, p53 is usually inactive and bound to the mdm protein (HDM2 in humans), which promotes its ubiquitination (binding with ubiquitin and degradation by proteasome) preventing its action. Triggered by damaged DNA (e.g., in ageing or ionizing radiation conditions), *p53* promotes a programmed cell death by arresting cell cycle at the G1 to S point and thus inhibits cellular proliferation and growth.

Mutations or loss of *p53* are a rather early event in pancreatic carcinogenesis and occur sporadically in most patients.[5,16,25,26] Specific mutation of p53 (R172P) has recently been associated with increased metastatic potential in pancreatic cancer models *in vitro*.[27] Additionally, *p53* mutations have been associated with reduced chemotherapy efficacy due to impaired *p53*-induced apoptosis.[28-30] Nevertheless, the prognostic significance of *p53* alterations remains unclear. The data is conflicting as few researchers have suggested a short survival in pancreatic cancer patients with *p53* mutations while others have found no association at all.[4,6,31-34]

4) *DPC4*

The tumor suppressor gene *DPC4* (deleted in pancreatic cancer, locus 4) or commonly called *SMAD4* has been long associated with pancreatic cancer. Genes of the

SMAD family encode for proteins that participate in tissue growth factor-beta (TGF-β) mediated signal transduction and thus regulate gene transcription and growth arrest. In particular, TGF-β binds to TGF-βRII receptor which subsequently activates TGF-βRI by phosphorylation. The signal transduction cascade also involves activation of TGF-βRI, phosphorylation and activation of *SMAD2* and 3 and finally formation intracellularly of a heterodimer complex with SMAD4.[35,36] This SMAD complex translocates to the nucleus and interacts with DNA where it controls transcription of genes, such as *c-myc*, *p21*, and *p15* which regulate cellular proliferation. The ultimate effect of SMAD4 in the normal cells will be growth arrest, apoptosis and cell differentiation by inhibition of the cell cycle at G1 point.

In pancreatic cancer, inactivation of *SMAD4* by point mutations or loss of heterozygosity (LOH) allows uncontrolled cellular growth and proliferation. This is a likely late event of pancreatic carcinogenesis as the gene is expressed normally in the early PanIN1 and 2 stages but only in a third of PanIN3 cases.[10,37]

Up to half of pancreatic cancer patients carry the inactivated *DPC/SMAD4* gene.[9,37] According to a recent published work, DPC4 immunolabelling may be of diagnostic value as it can possibly differentiate pancreatic metastatic disease from primary liver, lung or ovarian neoplasms.[38] Whether the *DPC4* status has a prognostic value remains subject of debate. In a few studies, presence of *DPC4* status was associated with better outcome and survival post resection,[10,39] but in other studies *DPC4* expression was associated with worse outcome after surgery or adjuvant chemotherapy.[40,41] Recent data has suggested that DPC4 loss is associated with presence of widespread metastases but it is not as frequently found in locally advanced pancreatic cancer, therefore may have a role in patients' selection for systemic rather than local treatment.[42]

Another effect of *DPC4/SMAD4* is reduction of angiogenesis by decreasing vascular endothelial growth factor (VEGF) and increasing thrombospondin (an anti-angiogenetic factor) expression. It was found that restoration of SMAD4 loss in pancreatic cancer cells resulted in slowly growing tumors with reduced vascular density, suggesting a possible tumor suppression mechanism.[43]

5) *BRCA2*

BRCA2 (breast cancer type 2) is a tumor suppressor gene, mutations of which are often associated with familial breast and ovarian cancer, and less often with other neoplasias and hematological diseases. It is located in chromosome 13q and its main function is normally the repair of damaged DNA.

Specific germline mutations of *BRCA2* gene, mainly at locations 6174delT and 6158insT, have been found in 6-17% of familial pancreatic cancer.[44-47] The same germline mutations were seen only in 10% of sporadic cases.[48,49]

The prognostic or predictive value of *BRCA2* in pancreatic cancer is still unknown.

6) *Erb* family genes (HER-2/neu - EGF)

The proto-oncogene *HER-2/neu* or *ErbB2* with its protein HER-2/neu is one of the four members of the epidermal growth factor receptor family (ErbB protein family) which regulate signal transduction from extracellular stimuli to the nuclear level. The relevant pathway is primarily the phosphatidylinositol-3 kinase and mitogen-activated protein kinase (PI3/Akt-MAPK) pathway though interference and cross-talk with other pathways is often taking place. Overexpression of proto-oncogenes or loss of tumor suppressor genes of the aforementioned pathways lead to unbalanced signal transduction and uncontrolled proliferation often seen in many neoplasias including pancreas.

In pancreatic adenocarcinoma, the rate of amplification or overexpression of the *HER-2/neu* gene varies in different studies from 16% to 65%.[50-53]

Most importantly, it seems that *HER-2/neu* gene alterations bear no prognostic or predictive significance.[8,54] Targeting HER-2/neu with monoclonal antibodies has been studied in preclinical and clinical setting with unclear results as we will see later in this paper.

7) *Notch1* and *Hedgehog*

Notch1 is a gene located at chromosome 9q which is normally involved in cell differentiation, proliferation and apoptosis. Notch signaling pathway seems to play role in embryogenesis and to regulate epithelial stem cells differentiation, survival and cell fate. Additionally, Notch interacts with the molecular pathways of Wtn and Hedgehog (Hh) in order to control proliferation of stem cells and cellular differentiation. Recent studies have suggested that sustained activation of these pathways might be related to cancer stem cells initiation and carcinogenesis. Likely, this is achieved through induction of nuclear factor-kappa B (NF-κB) and its signaling pathway by Notch. Persistent activation of NF-κB is very often found in pancreatic cancers and its role is increasingly recognized. Down-regulation of notch-1 and inactivation of NF-κB by natural products and phytochemicals such as curcumin and genistein or small interfering RNA resulted in inhibition of cancer progression and metastases.[55-58]

Currently, these natural compounds are tested in clinical trials in combination with conventional chemotherapy in patients with advanced pancreatic cancer. Increased expression of notch-1 was also noted in the intratumoral nerves of pancreatic cancer cell lines and it was associated with an invasive and angiogenic phenotype of pancreatic cancer *in vitro*. These findings suggest that notch pathway may regulate the neurovascular development of pancreatic cancer, but most importantly may be a therapeutic target in future.[59] As far as sonic Hh is concerned, while it normally promotes pancreatic cells differentiation, in pancreatic cancer SHH signaling pathway is often dysregulated, promoting tumour progression by increasing desmoplasia and facilitating recruitment of fibroblasts which in turn contribute to tumour-stromal cells interaction. Targeting of SHH pathway and the associated desmoplasia, e.g., with neutralizing antibodies or by blocking the SHH receptor Smoothened (SMO) with small interfering RNA, may be a valuable treatment option in future and needs to be further explored.[60,61]

8) COX pathway

Cyclooxygenase (COX) pathway has long been investigated and targeted in pancreatic cancer patients. COX is an enzyme which converts arachidonic acid to thromboxanes and prostaglandins. We find COX in two isoforms, COX-1 and COX-2. COX-1 is constitutively and naturally expressed in most tissues. On the contrary, COX-2 is mainly induced by cytokines and inflammatory stimuli but also by growth factors and oncogenes. Overexpression of COX-2 has been observed and implicated in the carcinogenesis of most solid tumors, including pancreas.[62]

Overexpression of COX-2 is a poor prognostic factor in pancreatic cancers.[63-66] The development of specific COX-2 inhibitors (celecoxib, apricoxib) has led to their investigation in clinical trials in advanced pancreatic cancer in combination with cytotoxic treatment and in the chemoprevention of pancreatic cancer.

9) Other genes

Akt-2 oncogene is implicated in pancreatic carcinogenesis and is often amplified and overexpressed in pancreatic tumors.[67,68] It has been proposed that inhibition of *Akt-2* results to decreased activity of NF-κB, lower levels of the anti-apoptotic gene *bcl-2* and increased levels of the pro-apoptotic gene *Bax*. Similarly, inhibition of *Akt-2* rendered cancer cells more sensitive to chemotherapy-induced apoptosis.[69]

Other genes involved in development of pancreatic cancer include *cyclins D1* and *D3*, which are regulating cell cycle at the G1/S point. These genes, which are often overexpressed in pancreatic cancer, have also been associated with poor prognosis.[70-72]

Finally, a possible role in pancreatic carcinogenesis, tumor progression and metastasis, but with limited evidence, may be played by genes *MUC4*, *Scr*, *Bcl-6*, *mdm2* and *S100P*.[26,73,74]

10) Latest identified altered molecules

(1) Palladin: This is an actin-associated protein that was found mutated in familial cases of pancreatic cancer and overexpressed in many sporadic pancreatic tumors and premalignant stages. There are two isoforms of this protein (65 kDa and 85 kDa), each of which is associated with specific properties and behaviour of tumor cells. Though some recent data suggests that this protein isoforms may be candidate biomarkers for early diagnosis and prediction of metastatic potential, other studies provided inconsistent conclusions about its role. Therefore, more research on this molecule is needed prior to any clinical application.[75,76]

(2) Micro-RNAs: These small non-coding RNA molecules control the activity of one third of all protein-coding genes. Altered expressions of miRNAs are implicated in carcinogenesis of various cancers by affecting apoptosis and cell growth. Deregulation of miRNA has been studied in pancreatic cancer in terms of cancer development and progression. There is preclinical evidence that specific miRNAs, including miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b, and miR-95 are upregulated in pancreatic cancer cells and involved in its pathogenesis.[77] Few of these mi-RNAs, such as miR-210, miR-200a and miR-200b which promote carcinogenesis through expression of E-cadherin, may be found elevated in the serum of patients with pancreatic cancer and may therefore be used in future for diagnostic purposes.[78-80]

2. Molecular pathways involved

Similarly to other solid tumors, some of the complex molecular and signaling pathways that are altered in pancreatic cancer have been recognized and efforts to repair identified abnormalities are mounting in drug development units. One of the central molecules in transduction pathways is NF-κB. NF-κB represents a family of molecules in the cytoplasm which upon binding to proteins IkBa and p100 becomes inactive. The NF-κB family contains five members, p50, p52, p65, c-Rel and RelB, which form and appear in heterodimers. Activation of NF-κB is achieved by phosphorylation of its binding proteins IκBα by IKKβ and/or p100 by IKKα which causes degradation of IκBα and transformation of p100 into the small form p52. Consequently, the active heterodimers of NF-κB

(the p50/p65 and p52/RelB) translocate to the nucleus where they bind to NF-κB-specific DNA-binding sites and to gene promoters regulating their transcription and expression. Known genes regulated by NF-κB include *survivin*, *VEGF*, *EGF*, and *MMP-9* which in turn affect cellular survival and apoptosis but also tumor progression, invasion and metastasis.

In pancreatic cancer, NF-κB is overactivated contributing to its pathogenesis, its local progression and distal spread.[55,56] Furthermore, activation of NF-κB by gemcitabine has been implicated as a potential mechanism of resistance to this drug.[81] NF-κB related pathway has recently been a target of novel agents tested in cancer research.

The Hh signalling pathway has been found to play a role in pancreatic carcinogenesis as stated previously. Hh pathway is overexpressed in up to 70% of pancreatic cancers.[82] Cyclopamine is a natural inhibitor of Shh able to induce apoptosis and inhibition of pancreatic cancer cell proliferation in cell lines and *in vivo*.[83] There is evidence of cross-talk between hedgehog pathway, NF-κB and notch-1. It seems that activation of NF-κB causes overexpression of Shh and accordingly inhibition of NF-κB may cause down-regulation of Shh.[84]

Other altered molecular pathway involved in pancreatic carcinogenesis is the RAS/RAF/MEK/MAPK pathway. The frequently mutated *KRAS* gene encodes for a constitutively activated KRAS protein causing up-regulation of the downstream molecules and uncontrolled cellular proliferation and survival.

Matrix metalloproteinases (MMPs) play in general significant role in cancer progression, invasion and metastases *via* extracellular matrix and stroma degradation facilitating distal migration of cancer cells. The main MMPs involved in pancreatic cancer are MMP-2 and MMP-9.[85] The degree of MMP-2 expression correlates with disease progression and poor prognosis.[86] It seems that the level of expression of the tissue inhibitor of metalloproteinases and its ratio to MMP-2 and -9 may be a prognostic factor of this disease and its metastatic potential.[85]

MOLECULAR TARGETS AND DIAGNOSIS

The disappointing outcome of pancreatic cancer patients, the majority of which are diagnosed at an advanced stage, necessitates the improvement of diagnostic tools and methods in order to identify more patients as early as possible. Thus, molecular targets are sought and the most valid are presented below.

1. Glycoproteins

1) CA-19-9

Tumor-associated antigen CA19-9 is a glycoprotein often produced by gastric and pancreatobiliary tumors. Its main utility is rather treatment monitoring and detection of disease recurrence than screening and initial diagnosis. The sensitivity and specificity for pancreatic tumors are 85 and 90%, respectively.[87,88] A number of other conditions including liver-biliary cirrhosis, biliary obstruction and ascites may account for increased CA19-9. For these reasons, the American Society of Clinical Oncology (ASCO) on its 2006 Update of Recommendations for the use of tumor markers in gastrointestinal cancer advised against the use of CA19-9 as a screening or diagnostic marker of pancreatic cancer.[89]

2) Mucins

Mucins (MUC) are the second most known glycoproteins studied in pancreatic tumors, characterized by their high molecular weight. Around twenty genes control and encode fourteen mucin proteins, which are linked individually with a particular pancreatic histological subtype.[90,91] Of particular interest is *MUC1* which is highly expressed in invasive ductal carcinoma, and less important for this review article is *MUC2* which is expressed in Intraductal Papillary Mucinous Neoplasm (IPMN) of dark cell type and *MUC6* found in clear cell type IPMN.[91,92]

MUC1 interferes normally to the intracellular and cell to stroma interaction, as well as inhibits the signal transduction in tumors and finally the cancer progression. There is evidence that MUC1 play a role in diagnosis and differential diagnosis of pancreatic cancer.[91,93] According to a recent meta-analysis of 17 studies (1,363 patients in total) regarding the role of MUC1 as a diagnostic tool in pancreatic adenocarcinoma, the accuracy of the test showed a sensitivity of 0.83 (95% CI, 0.81 to 0.86), specificity 0.63 (95% CI, 0.59 to 0.66) and diagnostic odds ratio of 20.44 (95% CI, 9.53 to 43.85). For these reasons MUC1 could be a potential test with moderate diagnostic accuracy in pancreatic adenocarcinoma.

MUC4 is another member of the *MUC* genes which has been found overexpressed in pancreatic cancer but not in benign conditions and therefore may be used as a potential diagnostic marker.[73] Overexpression of MUC4 is a poor prognostic factor and is associated with advanced stage of pancreatic cancer and aggressiveness.[94-96] Preclinical data from pancreatic cancer mice models suggest that inhibition of MUC4 with an antisense MUC4 RNA causes significant suppression of cancer growth and metastasis.[97]

Therefore, MUC4 needs further exploration as both a diagnostic marker and therapeutic target.

MOLECULAR TARGETS FOR TREATMENT

We presented above evidence on the main biological alterations implicated in pancreatic cancer pathophysiology. Treatment strategies and rationale of drug development is actually based on these molecular changes aiming to counteract the chief abnormal stimuli driving tumors (Table 1). Therefore, the main targeted agents which have been tested in pancreatic cancer include molecules against the EGFR, the HER2/neu receptor, MMP, the VEGF, the mTOR pathway, molecules against the activated KRAS

Table 2. Phase III and Some of the Phase II Clinical Trials of Targeted Agents in Pancreatic Cancer That Are Currently in Progress

Agents *(target)*	Clinical setting	Trial design	Treatment arms	Primary endpoints
Sorafenib	LAPC, metastatic (met.)	Phase III, RCT	Gem Gem+Sorafenib	PFS
Masitinib *(c-kit)*	LAPC, met.	Phase III, RCT	Gem+Placebo Gem+Masitinib	Overall survival (OS)
Erlotinib [E]	Resectable, adjuvant	Phase III, RCT 4-arms	I. Gem (5 cycles) II. Gem+E (5 cy) III. I or II+1 cycle IV. III+RT (5 wk)	OS in Gem +/− E group OS in Gem +/− RT group
Erlotinib [E], Sorafenib [S]	Unresectable PC	Phase II, single arm	E+S	PFS at 8 wk
GDC-0449 *(Shh)*	Recurrent (recur), met.	Phase II, D-blind, placebo controlled	Gem+Placebo Gem+DGC-0449	PFS
Cetuximab [C]	LAPC, met.	Phase II, single arm	Oxal+Irino+C	Efficacy
Panitumumab [P]	LAPC	Phase II, single arm	P+5FU-RT followed by P+G	Survival rate at 1 yr
Curcumin *(Nf-kB)*	LAPC, met., recur. PC	Phase II, single arm	Curcumin 8 gr/d	Survival, RR at 6 mo
Curcumin	Advanced PC, 1st line	Phase II, single arm	Curcumin+Gem	TTP
Sunitinib	Metastatic, maintainance after 6-mo chemotherapy	Phase II, randomized	Sunitinib Observation	PFS at 6-mo
Lapatinib [L]	LAPC, met. PC, 1st line	Phase II, single arm	L+Capecitabine	Survival rate at 6-mo
Lapatinib [L]	LAPC, met. PC, 2nd line	Phase II, single arm	L+Capecitabine	OS
Bevacizumab [B]	LAPC	Phase II, single arm	B+Gem+Oxal→ B+Oxal+5FU-RT	RR & TTP pre- and post-RT PFS, OS
Genistein *(Nf-kB)*	Resectable PC, neoadjuvant	Phase II, randomized	Genistein for 2 wk Observation	Changes in density of tumor microvessels
Erlotinib [E]	Metastatic PC	Phase II, single arm	E+Gem+Cisplatin	RR
Erlotinib [E]	Advanced PC, 1st line	Phase II, single arm	E+Gem+Oxal	RR
Erlotinib [E], Sorafenib [S]	Metastatic PC	Phase II, single arm	E+S+Gem	PFS at 4-mo
Sunitinib	Advanced PC, 1st line	Phase II, randomized	Gem Gem+Sunitinib	TTP
Bortezomib [Bor], Panobinostat [Pan]	Metastatic PC, Gem-resistant	Phase II, single arm	Bor+Pan	PFS
Erlotinib	Resectable, perioperative	Phase II, single arm	E (1 wk)→Surgery→ E+gem (6 mo)	Effect on predictive biomarkers
Cetuximab [C]	LAPC, unresectable	Phase II, single arm	C+RT (PACER)	PFS at 6-mo
Erlotinib	Resectable, adjuvant	Phase II, single arm	E+Capecitabine/RT→ E+Gem (4 mo)	PFS
Cetuximab [C]	LAPC, unresectable	Phase II, randomized	Gem-Cape (3 mo)→ UFT/LV+RT +/− C	OS at 1 yr
Pazopanib	Metastatic, 1st line	Phase II, single arm	Pazopanib+Gem	RR
Bevacizumab [B]	Advanced PC, 1st line	Phase II, single arm	B+Gem+5FU	PFS rate at 6-mo

Source: www.ClinicalTrials.gov.

LAPC, locally advanced pancreatic cancer; RCT, randomized controlled trial; Gem, gemcitabine; PFS, progression free survival; RT, radiotherapy; PC, pancreatic cancer; TTP, time to progression; RR, response rate; Oxal, oxaliplatin; UFT/LV; uftoral/leucovorin.

protein (farsenyl transferase inhibitors [FTI]) as well as many other agents which will be discussed later in our review. Various combinations of targeted agents with each other or with chemotherapy or radiotherapy are currently under investigation, the results of which will be available over the next few years (Table 2).

1. Targeting the EGF pathway

The EGF pathway and its molecules have been found altered quite often in pancreatic cancer. Thus, many well designed studies have been conducted trying to demonstrate some benefit from blocking the aberrant signal at various levels of the pathway.

Of particular interest are the inhibitors of EGF receptor. Two types of inhibitors against the EGFR exist. First, monoclonal antibodies (MAbs) against the extracellular part of the receptor and second small molecules against the tyrosine kinase part intracellularly (TKIs).

1) Positive studies

The only biological compound that showed positive results in a phase III study in combination with chemotherapy in the treatment of advanced pancreatic cancer is erlotinib. Erlotinib (Tarceva™) is a tyrosine kinase inhibitor (TKI), available in oral preparations, that blocks selectively the EGF receptor. Early preclinical studies had showed its potential to inhibit the EGF and the MAPK (ERK1/2) pathway in pancreatic cancer models, enhancing cancer apoptosis in combination with gemcitabine and wortmannin (a PI3K inhibitor).[98]

Thereafter, many clinical studies demonstrated the satisfactory safety profile of this biological agent in pancreatic cancer patients and its potential efficacy in combination with gemcitabine, the most active cytotoxic in these patients till then.

As a result, a phase III, randomized, placebo controlled, study published in 2007 tested the efficacy of the combination erlotinib plus gemcitabine versus gemcitabine monotherapy.[99] This important study recruited 569 patients in total and demonstrated a statistically significant survival benefit (6.24 months vs 5.91 months, p=0.038) in patients who received the combination treatment as compared to patients on the gemcitabine arm. Though the absolute benefit was only of a few weeks, it was for a first time that a survival benefit from a novel agent was clearly demonstrated in a phase III study. Besides, the 1-year survival rate was better in the combination arm (24% vs 17%, p=0.023). In subsequent subgroup analysis of this study, a particular survival benefit was demonstrated in those patients on erlotinib arm who developed high grade skin rash (≥2 according to the NCI Common Terminology Criteria for Adverse Events, version 3.0) providing thus a clinical biomarker of efficacy.[99,100] It was observed that the higher the degree of rash, the better the disease response and survival.[100] The recommended dose of erlotinib in this study was 100 mg per day.

In order to further improve the modest results, combinations of erlotinib with other agents have been under evaluation. A phase I/II study of erlotinib with bevacizumab, capecitabine and gemcitabine in chemotherapy naive patients with advanced pancreatic cancer, conducted in the United Kingdom, confirmed the safety of this combination and set the recommended dosing, according to the maximum tolerated doses, while showed promising efficacy justifying further exploration of the quadruple combination.[101,102]

Currently, clinical trials evaluating the combination of erlotinib/gemcitabine with MK0646 or cixutumumab (novel inhibitors of insulin growth factor receptor-1), sorafenib, GDC-0449 (hedgehog antagonist), apricoxib (selective COX-2 inhibitor), nab-paclitaxel or oxaliplatin are recruiting patients. Hopefully some of these trials will be positive providing further valuable treatment options.

2) Negative studies

Gefitinib (Iressa™) is a small molecule TKI which inhibits phosphorylation of the EGFR in the same way as erlotinib. Although, in pancreatic cancer cell lines there was evidence of antitumor activity, in a phase II on gemcitabine-resistant pancreatic cancer patients, combination of gefitinib with docetaxel, showed no actual clinical benefit.[103,104] On the other hand, combination of gefitinib with gemcitabine showed some activity (response rate of 11% and disease stability 23%) in advanced PC, according to a small phase II study by the Hellenic Cooperative Oncology Group in 2007, though the results have not been replicated since.[105] Lapatinib (Tyverb or Tykerb™) is a dual EGF (ErbB1) and HER2/neu (ErbB2) tyrosine kinase inhibitor. Recent phase II study of lapatinib and gemcitabine, in metastatic pancreatic patients, was terminated prematurely when an interim analysis, after 29 patients were recruited, showed that the combination was not effective.[106]

Apart from small molecules TKIs, quite a few monoclonal antibodies (mAbs) against the EGF family receptors have been tested in clinical trials in advanced pancreatic cancer patients, with disappointing so far results. Trastuzumab (Herceptin™) is a HER2/neu mAb known for its success in HER2/neu expressing breast cancers. Trastuzumab was also tested in PC patients as HER2/neu is often overexpressed in this disease.[50,51,53] In

contrast to some promising preclinical data, investigation of this agent in pancreatic cancer patients in a phase III study showed that despite its satisfactory toxicity profile and moderate activity, there was no survival benefit.[107] One possible explanation for this failure could be the fact that only 12% of the enrolled patients overexpressed (+3) HER2/neu receptor. **Cetuximab** (Erbitux™) is a chimeric antibody that inhibits the EGF receptor (ErbB1). Cetuximab has been studied extensively in both preclinical and clinical studies in pancreatic cancer. Based on positive evidence from tumor cell lines and animal models, this EGFR inhibitor was tested in clinical trials alone or in combination with chemotherapy.

Initial results from a phase II study showed that combination of cetuximab with gemcitabine in pancreatic cancer patients was feasible, well tolerated and moderately active.[108] Unfortunately, when the combination was tested in a large phase III study no survival benefit was found to justify further use.[109] Combinations of cetuximab with other cytotoxics or biologicals are under evaluation and results are anticipated in the years to come. For example, a phase II study of cetuximab along with oxaliplatin and irinotecan is currently recruiting patients in the United States. The combination sounds interesting bearing in mind the positive results from a retrospective analysis of patients treated with these drugs presented at the 2007 Annual Meeting of the ASCO and the very promising activity of FOLFIRINOX combination as presented in ASCO Annual Meeting in 2010.[110,111] As far as biologicals are concerned, combination of cetuximab with trastuzumab or everolimus is now under evaluation.

2. Targeting the VEGF pathway

Angiogenesis plays important role in cancer development and progression and VEGF is frequently overexpressed in pancreatic.[112,113] Therefore, there is a rationale of targeting the VEGF receptor with antibodies or small molecules often concurrently with other biological or cytotoxics. The most studied VEGFR inhibitor is the mAb **bevacizumab** (Avastin™). This is humanized antibody that blocks both VEGF receptors 1 and 2. As most of the biological tested in this devastating disease, bevacizumab showed evidence of activity in preclinical and early clinical studies. Sadly, when a subsequent double-blind phase III placebo controlled study was conducted, the combination of gemcitabine-bevacizumab showed no survival benefit compared to gemcitabine monotherapy.[114]

As stated before, combinations of bevacizumab with other biologicals and conventional chemotherapy drugs are currently tested in various clinical studies. Of promise are the results of the phase II study of bevacizumab with erlotinib, capecitabine and gemcitabine conducted at the Royal Marsden Hospital, United Kingdom presented at ASCO 2010 Annual Meeting though the toxicity is of some concern for patients with less good performance status.[102]

More molecules against the VEGF pathway are now available for testing in cancer patients including pancreatic cancer ones. Such molecules that are evaluated in trials include aflibercept, a recombinant fusion protein which is a potent inhibitor of VEGF (known as VEGF trap) and of placental growth factor and vatalanib, a small molecule TKI targeting selectively VEGF Receptors 1, 2, and 3.

Additionally, molecules with broad spectrum of activity are available and enriching our options for clinical trials in order to identify the most effective and less toxic combination. Multi-tergeted agents with at least preclinical evidence of activity include vandetanib (Zactima™), a small molecule that inhibits the VEGFR-2, but also VEGFR-3, ErbB1 (EGF) and RET kinase[115] and sorafenib (Nexavar™), an inhibitor of RAF kinase, PDGFR-beta, VEGFR-2,-3 and c-kit with antitumor activity against several cancers.

3. Targeting other pathways and molecules

1) Inhibitors of MMPs

Marimastat is the first MMP inhibitor studied in solid cancers, with broad activity against multiple MMP such as 1, 2, 3, 7 and MMP-9. Following early studies assessing activity in pancreatic cancer cells and safety in humans, two phase III studies on advanced PC patients evaluated marimastat or marimastat plus gemcitabine versus gemcitabine monotherapy and both failed to show any meaningful clinical benefit from the new agent.[116,117]

There are other MMP inhibitors developed in drug units, that have either failed to demonstrate activity in pancreatic cancer clinical trials, such as tanomastat (BAY 12-9566),[118] or have only been tested in preclinical trials such as Ro 28-2653, a selective oral inhibitor of MMP-2 and MMP-9.[119]

2) Farnesyl transferase inhibitors (FTIs)

As *KRAS* is found altered in the majority of pancreatic cancer patients, efforts to block this molecule with selective inhibitors have been made in the last decade.

Tipifarnib (R115777, Zanestra™) is such a selective inhibitor of farnesyl transferase, one of the several enzymes required for the function of p21 (RAS), RhoB and other proteins of this pathway, involved in cell survival and apoptosis. Farnesylation of RAS is mandatory for the

function of this protein and the signal transmission from the membrane receptors down to the intracellular proteins.

Contrary to many preclinical studies showing antitumor activity against pancreatic cell lines and xenograft models, tipifarnib failed to show clinical benefit either as monotherapy or in combination with gemcitabine in various phase II and a phase III study.[120-122] Similarly, negative results were produced from clinical studies of a second FTI, lonafarnib (SCH66336) which despite tumor suppression in human xenografts *in vivo*[123] and goog toxicity profile in phase I studies,[124,125] no actual benefit was reported in a phase II study on patients with advanced pancreatic cancer as compared to gemcitabine treatment (Proc Am Soc Clin Oncol 2001;20:abstr 608). The many aforementioned negative studies underpin the complexity of pancreatic carcinogenesis and the need for multimodal treatment approach.

3) COX-2 inhibitors

The evidence of COX-2 overexpression in pancreatic cancer has led to their investigation in chemoprevention and treatment of this disease, often in combination with cytotoxics. Celecoxib is a COX-2 inhibitor that showed in laboratory pancreatic cancer models that it is able to induce apoptosis and to inhibit angiogenesis, tumor growth and metastasis.[126,127] Celecoxib has also demonstrated synergistic effect was in combination with cytotoxics (gemcitabine or fluopyrimidines), radiotherapy[128,129] or with other agents such as erlotinib and curcumin.[130,131] A possible explanation of the enhanced antitumor effects is the increased down-regulation of Her2/neu, EGFR and COX-2 expression along with inactivation of NF-kB.

A number of phase II studies have tested the efficacy of celecoxib in various clinical settings in pancreatic cancer. Combination of celecoxib with protracted 5-FU infusion or capecitabine as second line chemotherapy in patients with advanced pancreatic cancer showed minimal activity (response rate, 9% to 12%) and moderate risk of gastrointestinal or haematological toxicities.[132,133] Subsequent small phase II study on 18 patients with advanced inoperable pancreatic cancer showed that combination of gemcitabine, irinotecan and celecoxib was quite active (18% partial response, 70% stable disease, overall survival 13 months) but at the cost of common grade 3/4 toxicities (neutropenia [50%], anaemia [39%], diarrhoea [17%], fatigue [17%]).[134] Similar results were recently reported by other researchers (20% partial response , median survival 18 months), and thus the combination merits exploration in a phase III study.[135] Nevertheless, the combination of celecoxib with gemcitabine and cisplatin did not demonstrate added clinical benefit in terms of median survival in other phase II trials on advanced pancreatic cancer patients.[136,137]

One of the concerns regarding the use of COX-2 inhibitors is the increasing risk of cardiovascular diseases, and for that reason patients at risk for heart diseases should be considered for COX-2 inhibitors cautiously.[138-141] Currently, a randomised phase III, double-blind, placebo controlled study of celecoxib, gemcitabine and curcumin is recruiting patients with advanced pancreatic cancer and the results will shed some light on the synergistic effect of this combination (www.ClinicalTrials.gov, NCT00486460).

4) NF-kB inhibitors

NF-κB is a pivotal pathway in signal transduction and its overactivation is implicated in the development of tumors. Inhibition of expression or activation of NF-κB by various agents has been studied in preclinical models and less so in clinical trials.

Curcumin, a natural antioxidant found in turmeric (curry), is a potent inhibitor of the overexpressed NF-κB pathway and has demonstrated activity against pancreatic cancer and other solid tumors in preclinical and clinical studies.[142,143] In addition, curcumin showed it could enhance the cytotoxic effect of gemcitabine, at least *in vivo*.[144] Likewise, the soy isoflavone genistein was able to downregulate NF-κB increasing thus, the antitumor activity of gemcitabine, cisplatin or erlotinib.[145-147] Inhibition of NF-κB activation by nafamostat, a serine threonine inhibitor, was able to improve the cytotoxic effect of gemcitabine in mice models with pancreatic cancer.[81]

A high number of natural products or synthetic compounds may inhibit NF-κB activation, but it will take long before proper clinical studies will determine their actual impact in pancreatic cancer.

5) mTOR inhibitors

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase protein that controls cell cycle, protein translation and cell growth by regulating RNA in nucleus, but also affects angiogenesis through the hypoxia inducible factor 1 (HIF-1). This important pathway is constitutively activated in pancreatic cancer cells.[148] The first inhibitor of mTOR, rapamycin, that was identified serpenditiously, was able to cause cell cycle arrest at the G1/S phase and inhibition of pancreatic cancer cell proliferation and cancer growth.[149]

Since then many synthetic analogues of rapamycin have been developed such as temsirolimus (Torisel™) and everolimus (Affinitor™) with similar preclinical activity on pancreatic cell lines.[150] Furthermore, inhibitors of mTOR

potentiated the activity of other drugs (gemcitabine or erlotinib) in pancreatic cancers *in vitro* and *in vivo*.[151,152] Nevertheless, temsirolimus lacks yet clinical evidence in pancreatic cancer patients (still tested in phase I studies in combination with gemcitabine) and everolimus was found inactive in gemcitabine-resistant patients.[153]

Many clinical trials testing combinations of everolimus with other agents (irinotecan, capecitabine, cetuximab, sorafenib) are currently accruing patients.

6) MEK and HDAC inhibitors

The high frequency of mutations in *KRAS* gene has led to the development of molecules able to block the aberrant signal transduction below the level of the dominant abnormality in the RAS→RAF→MEK→MAPK (mitogen activated protein kinase)→ERK (extracellular receptor kinase)→FOS pathway.

An early study on pancreatic cancer cell lines showed that the MEK inhibitor UO126 may induce cell cycle arrest and inhibition of cancer cells proliferation by up-regulation of the cyclin-dependent kinase inhibitor p27Kip1.[154]

The first MEK inhibitor tested in clinical trials was CI-1040. This agent was found well tolerated and safe in a phase I clinical trial,[155] but rather ineffective in pancreatic cancer patients according to subsequent phase II study on solid tumor.[156] Other MEK inhibitors evaluated at present for efficacy in pancreatic cancer patients include GSK1120212 and AS703026, the former in combination with everolimus and the later with gemcitabine.

Histone deacetylases (HDAC) are enzymes that deacetylate histone, the protein which binds around DNA interfering with gene transription and suppressing their expression. Balanced function between the HDAC and histone acetyltransferases (HAT) is required for normal cellular function. Inhibition of these enzymes by HDAC inhibitors aims to modulate gene transcription and to affect cell cycle progression, apoptosis and angiogenesis.[157]

There is considerable preclinical data regarding the inhibitory effects of HDAC inhibitors on pancreatic cancer cells. Among those inhibitors we find Trichostatin A (TSA), the synthetic SK-7041 and FR901228.[158-160] TSA also showed synergistic cytotoxic effect on pancreatic cancer lines when combined with irinotecan or gemcitabine.[161,162] Another important HDAC inhibitor is Vorinostat (Zolinza™) also known as suberoylanilide hydroxamic acid (SAHA). Combination of vorinostat with gemcitabine induced apoptosis and pancreatic cancer cells inhibition *in vitro*.[163]

These studies suggest that HDAC inhibitors may sensitise cancer cells resistant previously to chemotherapy. Of interest, combination of vorinostat with the biological agents 5-Aza-2'-deoxycytidine (inhibitor of DNA methylation) or the proteasome inhibitor bortezomib (Velcade™) enhanced apoptosis and inhibition of cancer growth.[164,165] There are about four phase I/II clinical trials in progress testing the safety, dosing and efficacy of vorinostat in nonmetastatic pancreatic cancer patients along with radiotherapy, fluoropyrimidines or a proteasome inhibitor. Finally, a novel HDAC inhibitor, panobinostat, is also investigated combined with bortezomib in a phase II study on gemcitabine-resistant pancreatic cancer patients.

7) Targeting SHH

Sonic hedgehog pathway is implicated in pancreatic carcinogenesis through enhanced proliferation and survival of pancreatic epithelial cells, reduced apoptosis and interactions with KRAS pathway.[166] Targeting SHH by selective inhibitors such as cyclopamine, SANT1, robotnikinin, IPI-269609 and Cur-61414 have been developed and showed that may halt tumorigenesis and progression of pancreatic cancer.[167,168] There is preclinical evidence that cyclopamine may augment the effect of chemotherapy, anti-EGFR therapy and radiotherapy and may suppress distal spread of pancreatic cancer cells.[169,170] Similarly, combined inhibition of SHH and mTOR signaling pathways with cyclopamine and rapamycin achieved to eliminate pancreatic cancer stem cells proposing a novel therapeutic target for an aggressive disease.[171] A pilot clinical study of the SHH inhibitors GDC-0449 combined with gemcitabine, in advanced pancreatic cancer patients, is now in progress planning to recruit 25 patients until 2011.

CONCLUSION

Early diagnosis and treatment of pancreatic cancer is possibly the only way to improve significantly the disappointing outcome of the majority of pancreatic cancer patients. Despite the development in technical facilities and translational research, no real progress in clinical terms has been achieved so far. What we seem to have learnt though, is the complexity of pancreatic cancer pathogenesis, the mechanisms of progression and of resistance to treatments. This knowledge allows us to focus our efforts in targeting specific abnormalities simultaneously, aiming to reduce treatment failures. The development of novel drugs is costly and time consuming. Nevertheless, new and high quality knowledge always follows the proof of concept pathway and hopefully new agents will pass successfully through the drug development pipeline and well designed clinical studies, even

should be the very tight.

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