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Biological Approaches to Therapy of Pancreatic Cancer

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Pancreatic cancer · Gene therapy · Inhibitors · Oncolytic virus · Immunotherapy

Abstract
Pancreatic cancer is a lethal disease and notoriously difficult to treat. Only a small proportion is curative by surgical resection, whilst standard chemotherapy for patients with advanced disease has only modest effect with substantial toxicity. Clearly there is a need for the continual development of novel therapeutic agents to improve the current situation. Improvement of our understanding of the disease has generated a large number of studies on biological approaches targeting the molecular abnormalities of pancreatic cancer, including gene therapy and signal transduction inhibition, antiangiogenic and matrix metalloproteinase inhibition, oncolytic viral therapy and immunotherapy. This article provides a review of these approaches, both investigated in the laboratories and in subsequent clinical trials.

Introduction

Pancreatic cancer remains one of the most difficult conditions to treat, with only about 10% of patients presenting with resectable disease suitable for potentially curative surgery [1]. Outcome has not improved substantially over the past 25 years, with overall 5-year survival remaining dismally poor at 5% [2]. Patients with locally advanced disease have a median survival (MS) of 6–10 months, whilst for those with metastatic disease it is only 3–6 months [3].

For many years, 5-fluorouracil (5-FU) has been the standard chemotherapeutic regime for pancreatic cancer. Gemcitabine became the new standard of treatment after the publication of a phase III trial in 1997, showing a modest survival advantage over 5-FU and better alleviation of disease-related symptoms [4]. Recent meta-analyses showed that gemcitabine-based combination chemotherapy has a significant survival benefit over gemcitabine alone in locally advanced and metastatic pancreatic cancers [5]. There is currently insufficient evidence to endorse the use of chemoradiation, followed by chemotherapy, over chemotherapy alone in locally advanced disease [1]. As these conventional treatments often have limited effect and substantial toxicity, a strong need exists for novel therapies. Biological approaches that target pancreatic cancer at a molecular level are rapidly evolving and represent promising strategies to treat this deadly disease (table 1).

Gene Therapy and Signal Transduction Inhibition

The genetics and pathogenesis of pancreatic cancer is one of the most complicated of the malignant diseases [6]. Multiple genetic mutations have been identified as the

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Table 1. Biological approaches to therapy of pancreatic cancer

Gene therapy and signal transduction inhibition
Antisense oligonucleotides
RNA interference
Dominant negative mutants
Small molecule inhibitors
Gene restoration
Suicide gene therapy
Antiangiogenic and matrix metalloproteinase inhibitors
Oncolytic viral therapy
Immunotherapy
Antibodies – inhibitory, immunotoxin, radioconjugate
Adoptive cell transfer
Cytokines and immunomodulators
Vaccines – DNA, peptide, whole cell, antigen-pulsed dendritic cells

Table 2. Genetic and molecular aberrations in pancreatic cancer

	Frequency, %
K-ras	75–90
EGFR	69
Akt2	20
Bcl-2	23
Bcl-xL	90
COX-2	44–90
Gastrin precursors and gastrin	23–91
CCK-B receptor	95
IGF-1R	64
FAK	48–75
Smad4	55
TβRI	1
TβRII	4
Shh	70
Notch3	69–74
CaSm (LSM1)	87
BIRC5 (survivin)	77–94
$p16^{INK4A}$ (MTS1)	85
pRb	6
p53	50–75
VEGF	93
c-Met-encoded HGF receptor	61–87
MUC1	90
Mesothelin	90–100
RAAG12	100
Telomerase	92–95
CEA	85–90

precursor to the development of pancreatic cancer, but none are mutually exclusive (table 2). Gene therapy involves the introduction of exogenous nucleic acids into cells. By expressing, restoring or inhibiting a particular gene of interest, it is hoped that this would prevent or reverse the growth of cancer cells. Complex tumour-promoting signalling pathways have also been identified and these can be targeted by molecular inhibitors.

Signal Transduction Pathways

Ras

The K-ras oncogene (homologous to the ras gene of Kirsten murine sarcoma virus) mutation, mostly at codon 12 but also occasionally at codons 13 and 61, occurs in 75–90% of pancreatic cancers [7]. The gene encodes a 21-kDa membrane-bound guanosine triphosphate (GTP)-binding protein involved in growth factor-mediated signal transduction pathways. It can be activated through the overexpression or activation of ras-activating signalling partners, such as the epidermal growth factor receptor (EGFR). The best-characterised effector pathway in ras function is the Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK; MEK) cascade. Mutations result in impaired GTPhosphatase (GTPase) activity, causing it to be locked in the GTP-bound state and thus activating downstream signalling cascades [8]. There is no significant difference in the prevalence of K-ras mutation at different stages of the disease [9], and they are often found in benign lesions of the pancreas [10], indicating that activation of this oncogene is involved in the initiation or early phase of carcinogenesis. Another less characterised member of the ras family, H-ras (homologous to the ras gene of Harvey murine sarcoma virus), also plays a role in promoting tumour growth. The H-ras-ERK cascade is activated by transforming growth factor-α (TGF-α) in pancreatic cancer cells with K-ras mutations [11].

Antisense gene therapy involves the administration of oligonucleotides with sequences complementary to regions of specific mRNA strands, resulting in the prevention of gene translation. Transfection with a plasmid encoding antisense K-ras RNA (AS-K-ras-LNSX) suppressed growth of several human pancreatic cancer cell lines and administration of this agent to nude mice bearing the pancreatic AsPC-1 peritoneal implants inhibited tumour progression [12]. N116Y is a dominant negative H-ras mutant, derived from the v-H-ras oncogene by substituting asparagine with tyrosine at codon 116, which is in the GTP-binding domain [13, 14]. N116Y prevents the activation of oncogenic ras protein with which it competes for a guanine nucleotide exchange factor [15]. An E1-deleted, replication-deficient adenovirus (Ad CEA-

N116Y), in which N116Y was driven by the human carcinoembryonic antigen (CEA) promoter, effectively reduced the number of PCI-43 hepatic metastases following intrasplenic injection [14]. In a phase II trial of the antisense inhibitor of H-ras ISIS-2503 in combination with gemcitabine, patients with locally advanced or metastatic pancreatic adenocarcinoma showed a response rate of 10.4% and an MS of 6.6 months [16] (5% and 5.7 months, respectively, for gemcitabine alone [4]).

RNA interference is a relatively new technology in comparison to the antisense system. The gene-silencing process of RNA interference involves the manufacture of short, double-stranded RNAs (<30 bp), termed small interfering RNAs (siRNAs), by the RNase enzyme Dicer. These siRNAs are then incorporated into a silencing complex called RISC (RNA-induced silencing complex), which identifies and silences complementary mRNA [17]. siRNAs targeting K-ras reduced proliferation of Panc-1 and MIAPaCa-2 pancreatic cancer cell lines, although only MIAPaCa-2 showed increased apoptosis [18]. Retroviral delivery of siRNAs inhibited the growth of Capan-1 cells, both in vitro and in vivo [19]. Inhibition of the mutant K-ras^{v12} was shown whilst other ras isoforms were unaffected, demonstrating the extraordinary specificity of siRNA as the wild-type and mutant ras differs only in a single codon.

Post-translational modification of ras protein involves the addition of a 15-carbon farnesyl isoprenoid moiety to the cysteine residue of the C terminus, mediated by farnesyl protein transferase. Farnesylation is necessary for ras to attach to the cell membrane. However, tipifarnib (R115777), a farnesyltransferase inhibitor (FTI), has been disappointing in a phase III study of 688 patients [20]. The MS for the gemcitabine plus tipifarnib arm was 193 compared with 182 days for gemcitabine plus placebo ($p = 0.75$), with no difference in 1-year survival and progression-free survival (PFS). Possible explanations include the fact that although H-ras is exclusively modified by FT, K-ras and N-ras can also be modified by geranylgeranyltransferase (GGT) [21]. This provides an alternative route to the creation of biologically active ras. Furthermore, FTI works by inducing cell cycle arrest, whereas gemcitabine requires cell cycle progression to be effective [22]. Nevertheless, FTI and siRNA inhibition of FT have been found to increase the sensitivity of pancreatic cancer cell lines to radiation [22, 23]. FTI and GGTI in combination enhanced tumour apoptosis in mice bearing the pancreatic tumour PSN-1 despite significant toxicity [24]. L-778,123, a dual inhibitor of FT and GT, has been tested in a phase I trial in combination with radiotherapy [25]. Eight of the 12 patients experienced no dose-limiting toxicities on the lowest dose, with one of them showing a partial response of 6 months' duration. Reversible farnesylation and radiosensitisation were demonstrated in a patient-derived cell line.

Epidermal Growth Factor Receptor

The EGFR (also known as human EGF receptor 1 – HER1 or ErbB1) is a transmembrane glycoprotein with an intracellular tyrosine kinase domain. Binding of ligands to EGFR causes receptor homodimerisation or heterodimerisation, leading to phosphorylation of tyrosine residues on the intracellular domain, activating a downstream signalling cascade, including MAPK (cell proliferation), PI3K/Akt (cell cycle progression and survival), and the signal transducer and activator of transcription (STAT) family of proteins (cell division, survival, motility, invasion and adhesion) [26]. Mechanisms that lead to aberrant receptor activation include receptor overexpression, gene amplification, activating mutations, overexpression of receptor ligands, and/or loss of their negative regulatory mechanisms [27, 28]. EGFR and its ligands EGF and TGF-α are overexpressed in pancreatic cancer [29–31], and are associated with tumour aggressiveness [32].

Erlotinib (Tarceva or OSI-774) is an orally active small molecule that binds to the adenosine triphosphate binding site on the intracellular tyrosine kinase domain of EGFR. EGFR transactivation of HER3 (ErbB3) mediates Akt signalling, and that coexpression of HER3 with EGFR contributes to erlotinib sensitivity for pancreatic tumours [33, 34]. Administration of an EGFR tyrosine kinase inhibitor (PKI166) in mice showed inhibition of tumour-induced angiogenesis and increased apoptosis of tumour-associated endothelial cells [35]. A phase III trial has been completed recently for erlotinib in combination with gemcitabine in 569 patients with advanced pancreatic cancer [36]. MS in the erlotinib/gemcitabine arm was better at 6.24 compared to 5.91 months in the placebo/gemcitabine arm, with 1-year survivals of 23 and 17%, respectively ($p = 0.023$). More patients on erlotinib had disease stabilisation. This was the first trial to show a survival benefit, and the United States Food and Drug Administration has already approved the use of this combination in 2005. European registration is restricted to those with metastatic but not locally advanced disease. The most frequent toxicities are diarrhoea and rash, with the latter being associated with better outcome. In patients with gemcitabine-refractory advanced pancreatic cancer, treatment with capecitabine and erlotinib was as-

sociated with an overall objective radiologic response rate of 10% and an MS of 6.5 months [37].

Gefitinib (Iressa or ZD1839) is another EGFR tyrosine kinase inhibitor that also inhibits anchorage-independent growth and invasiveness of pancreatic cancer cells [38]. Sensitivity to gefitinib is correlated directly with ligand (TGF-α) expression [39]. A phase II trial combining gemcitabine with gefitinib in patients with inoperable or metastatic pancreatic cancer has shown results similar to those of gemcitabine with erlotinib [40]. However, phase II trials of capecitabine or docetaxel with gefitinib as second-line therapy for patients with advanced pancreatic cancer were unimpressive [41–43]. Lapatinib (GW572016) has shown promising phase I results [44, 45] and is now being tested in a phase II trial with gemcitabine.

MEK

As discussed above, the Ras/Raf/MEK pathway plays a central role in mediating the transmission of growth-promoting signals in pancreatic cancer. ARRY-142886 (AZD6244), an orally active inhibitor of MEK-1 and MEK-2, caused tumour regression in a human pancreatic cancer cell line BxPC-3 xenograft model [46]. Another inhibitor, CI-1040 (PD184352), was tested in a phase II study of 67 patients (15 with pancreatic cancer) [47]. No significant antitumour activity was demonstrated.

PI3/Akt Pathway

The phosphatidylinositol-3-kinase (PI3K)/Akt pathway plays a role in cell proliferation, survival and resistance to apoptosis [48, 49]. Class 1A PI3Ks are composed of heterodimers of an inhibitory adaptor/regulatory (p85) and a catalytic (p110) subunit [50]. Akt2 (also known as protein kinase B-β), the human homologue of the viral oncogene v-akt, is amplified in 20% of pancreatic cancers and its suppression by antisense RNA reduced growth and tumourigenicity [51, 52]. Upon activation by EGFR or ras, PI3K activates Akt which in turn has multiple downstream targets, including mammalian target of rapamycin (mTOR) which regulates cell growth and metabolism in response to environmental cues [53], and the transcription factor nuclear factor κB (NFκB) which promotes cell growth, angiogenesis and invasion [54, 55]. Phosphatase and tensin homologue deleted from chromosome 10 (PTEN) dephosphorylates the 3′OH group phosphorylated by PI3K, acting as a tumour suppressor.

PI3K inhibitors such as wortmannin and LY294002 induced apoptosis of pancreatic cancer cell lines that display constitutive Akt phosphorylation (Panc-1, MIAPaCa-2, HPFA, Colo-357-L3.6pl, Capan-2 and CFPAC-1), but not in cells that did not (AsPC-1, BxPC-3 and Hs766T). In vivo exposure of Colo-357-L3.6pl in nude mice to LY294002 led to the inhibition of tumour growth and reduction in hepatic metastases [56]. Wortmannin also showed significant antitumour effect in a PK1 murine xenograft model when combined with gemcitabine [57].

RASN17 is a dominant negative mutant of ras that inhibits PI3K/Akt pathway upstream of PI3K, whilst AAA-AKT is a dominant negative mutant of Akt. Overexpression of these inhibitors induced apoptosis and abolished anchorage-independent growth of pancreatic cancer cells. Adenoviral vector carrying RASN17 also produced significant antitumour effect in vivo in mice bearing HPAF tumours [58].

The mTOR inhibitor temsirolimus (CCI-779) was found to have a significant antitumour effect in vivo when combined with gemcitabine, both in AsPC-1 subcutaneous and SUIT-2 peritoneal dissemination xenograft models [59]. Everolimus (RAD001) is an mTOR inhibitor that also downregulates the antiapoptotic protein survivin. Given before survivin siRNA, there was enhanced cell death in vitro in the pancreatic cancer cell lines MIAPaCa-2, BxPC-3, AsPC-1 and Panc-1 [60]. Sirolimus (rapamycin) was shown to be active against mTOR, hypoxia-inducible factor-1α and vascular endothelial growth factor (VEGF), and inhibited the growth of SW1990 human pancreatic cancer cells in vivo [61]. These are currently being tested in phase II trials.

Proteosomes are enzymes that degrade multiple intracellular proteins, such as IκB (an endogenous inhibitor of NFκB), cyclin-dependent kinase inhibitors p21 and p27, cyclins and p53 [62]. Bortezomib (Velcade or PS-341) is a proteosome inhibitor that promotes apoptosis [63, 64] and increases cell sensitivity to chemotherapeutic agents [65]. Given after gemcitabine it resulted in significant induction of apoptosis and long-term growth inhibition of MIAPaCa-2 cells in vitro [66]. Given in vivo it enhanced the effect of docetaxel on MIAPaCa-2 and L3.6pl by cell growth arrest and inhibition of angiogenesis [67]. Bortezomib in combination with irinotecan showed impressive antitumour activity in mice bearing BxPC-3 xenografts [68]. When used in conjunction with HA14-1, an inhibitor of Bcl-2 (an antiapoptotic protein expressed in 23% of invasive ductal adenocarcinomas of the pancreas [69]), increased oxidative stress and apoptosis were noted on BxPC-3 cells [70]. Completed phase I trials of bortezomib in combination with chemotherapy for solid tumours have shown promising results [71–73]. Another

oral proteosome inhibitor, NPI-0052, resulted in significantly enhanced antitumour response when combined with either gemcitabine or cetuximab in a Panc-1 murine xenograft model [74].

Genistein is a naturally occurring isoflavone present in soybeans, and is believed to protect people who consume a diet high in soy products from pancreatic cancer. It inhibits cancer cell growth and angiogenesis, induces apoptosis, and chemosensitises cells by inhibition of PI3K/Akt/NFκB and Notch signalling [75–79]. Pre-treatment of pancreatic cancer cells with genistein followed by either gemcitabine [80] or cisplatin [81, 82] potentiated the effect of the chemotherapeutic agent both in vitro and in vivo. Significant growth inhibition and apoptosis were also demonstrated when genistein was combined with erlotinib in BxPC-3, Capan-2 and AsPC-1 cells, whilst in Colo-357 cells the addition of gemcitabine (triple therapy) significantly improved treatment efficacy [83]. As one of the functions of NFκB is to activate Bcl-xL (an anti-apoptotic protein expressed in 90% of invasive ductal adenocarcinomas of the pancreas [69]), it has been shown that pre-treatment of pancreatic cancer cells in vitro with genistein followed by the Bcl-xL inhibitor BL-193 significantly inhibited cell growth [84].

Curcumin (diferuloylmethane), derived from the spice turmeric *(Curcuma longa)*, is a non-toxic agent that inhibits NFκB and therefore the expression of NFκB-regulated gene products, including Bcl-2, Bcl-xL, cyclooxygenase-2 (COX-2), cyclin D1, VEGF, survivin, interleukin-8 (IL-8) and matrix metalloproteinase-8 (MMP-8) [85, 86]. Curcumin was able to inhibit proliferation and induce apoptosis in pancreatic cancer cells in vitro [85, 87, 88]. The antitumour activity of gemcitabine was also potentiated by curcumin, both in vitro and in vivo (orthotopic mouse model with MIAPaCa-2 cells) [85, 89]. ApoG2, a small molecule inhibitor of Bcl-2 and Bcl-xL, showed enhanced antitumour effect in BxPC-3 cells after pre-treatment with curcumin [90]. In a phase II trial of patients with advanced pancreatic cancer, oral curcumin was well tolerated, and although it has limited absorption, it was able to decrease the expression of tumour-promoting molecules including NFκB, COX-2 and phosphorylated STAT-3 in peripheral blood mononuclear cells. Of the 21 evaluable patients, one had a brief marked tumour regression [91]. A phase II trial with gemcitabine is ongoing.

Cyclooxygenase

The COX enzymes, COX-1 and COX-2, function to convert arachidonic acid into prostaglandins. COX-1 is constitutively expressed in normal tissues and plays a role in tissue homeostasis such as in the kidneys, gastric mucosa and platelets. COX-2 is inducible by growth factors, cytokines and tumour promoters [92]. It is upregulated in 44–90% of pancreatic cancers [93–95]. The molecular mechanisms of COX- and prostaglandin-mediated pancreatic cancer development are complex, involving multiple mitogenic signalling pathways and molecules including PI3K/Akt, STAT, ERK1/2, tyrosine kinases and NFκB; regulators of cell migration, invasion and angiogenesis such as MMP-2 and VEGF; immune suppression by prostaglandins, and the production of free radicals and peroxidation of procarcinogens to carcinogens by COX-2 [92]. K-ras mutation is not correlated with COX-2 expression [96].

Evidence exists to suggest that COX inhibitors, or non-steroidal anti-inflammatory drugs (NSAIDs), can reduce the risk of developing pancreatic cancer [97]. NSAIDs were able to suppress proliferation of pancreatic cancer cell lines in vitro predominantly through cell cycle arrest and induction of apoptosis, as well as inhibit angiogenesis in vivo [96, 98–102]. The antiproliferative effect was enhanced by gemcitabine. Phase II trials using gemcitabine in combination with celecoxib (Celebrex) 400 mg twice daily, a selective COX-2 inhibitor, have shown promising results. For 20 evaluable patients with metastatic pancreatic cancer, the MS was 6.2 months and the 3-month survival rate was 72% [103]. In another study of 32 patients with locally advanced and metastatic disease, the reported MS was 9.1 months [104]. Celecoxib, gemcitabine and irinotecan were also tested in 20 patients with advanced pancreatic cancer [105]. Of the 17 evaluable patients, the MS was 13 months and the 1-year survival rate was 64%, associated with improvement of pain and quality of life and reduction in CEA and CA19-9. Celecoxib in combination with cisplatin and fixed-dose rate infusion of gemcitabine in 22 patients with metastatic disease did not show any clinical benefit [106]. In a separate phase II study of 20 patients with advanced disease progressing after gemcitabine, the combination of celecoxib and infusional 5-FU was well tolerated, induced two partial responses, significantly reduced CA19-9 in 3 of 9 evaluable patients, leading to an MS of 14 weeks [107]. Study of celecoxib with capecitabine as a second-line therapy revealed an MS of 16 weeks, with 7 of 19 patients having a >25% decrease in CA19-9 [108].

Curcumin was able to synergistically enhance the growth inhibitory effect of celecoxib in the P-34 pancreatic cancer cell line, mediated through the inhibition of COX-2 [109]. A phase III trial of gemcitabine, celecoxib

and curcumin is currently in progress for patients with locally advanced and metastatic pancreatic cancers.

Gastrin-Cholecystokinin Receptor Pathway

Gastrin is a peptide hormone secreted by G cells of the gastric antrum and duodenum, and it can act as a growth factor for gastric, colonic and pancreatic cancers [110, 111]. Cholecystokinin-B (CCK-B) receptor, gastrin precursors, and the fully processed amidated gastrin are expressed in 95, 55–91 and 23% of pancreatic cancers, respectively, but not in the normal pancreas [112]. Both CCK-B and its splice variant CCK-C receptors bind to CCK and gastrin [113]. BxPC-3 cells transfected with the antisense DNA for CCK-C receptor showed a 65% decrease in cell numbers compared to control ($p = 0.002$). When performed in vivo, tumours of treated nude mice were 75% smaller in volume and 83% reduced in weight ($p = 0.03$) [113].

Gastrazole (JB95008), a selective CCK-B receptor antagonist, was tested in two randomised controlled trials in patients with advanced pancreatic cancer [114]. In a trial of 18 patients using protracted venous infusion (PVI) gastrazole compared with PVI placebo, gastrazole significantly improved survival – MS of 7.9 and 4.5 months and 1-year survivals of 33 and 11%, respectively (log rank $p = 0.02$). In the other trial, 98 patients were randomised to compare PVI gastrazole and PVI 5-FU. There was no significant difference in survival between the two (MS of 3.6 and 4.2 months and 1-year survivals of 13.2 and 26.2%, respectively, log rank $p = 0.42$), but gastrazole caused less diarrhoea, stomatitis and hand-foot syndrome.

Insulin-Like Growth Factor and Focal Adhesion Kinase

Insulin-like growth factor (IGF) and its receptors have been extensively studied in various cancers such as colon, breast and prostate [115]. In particular IGF-1 receptor (IGF-1R), a transmembrane receptor tyrosine kinase, has anti-apoptotic and growth promoting effects, acting via multiple pathways including PI3/Akt, MAPK and the Janus kinase (JAK)/STAT3 pathways [116–118]. It can be inhibited by antibodies, antisense RNAs and dominant-negative mutants [119].

IGF-1R is overexpressed in 64% of pancreatic cancer cells [120]. Adenoviral vectors carrying either the IGF-1R dominant negative inhibitor (Ad-IGF-Ir/dn) or short hairpin RNA for IGF-1R (Ad-shIGF-Ir) inhibited pancreatic cancer growth both in vitro and in vivo, and there was increased sensitivity to chemotherapy- and radiation-induced apoptosis [121]. AMG-479, a human anti-IGF-1R antibody, was recently tested in combination with gemcitabine and EGFR inhibitors in vivo [122]. In MIAPaCa-2 xenografts, AMG-479 was more effective in suppressing tumour growth when combined with gemcitabine than with either agent alone. For BxPC-3, AMG-479 showed additive effect when combined with EGFR inhibitors erlotinib, gefitinib and panitumumab. Inhibition of the PI3/Akt pathway was demonstrated.

NVP-TAE226, a dual focal adhesion kinase (FAK) and IGF-1R kinase inhibitor, showed tumour suppressive effect on MIAPaCa-2 cells in vivo [123]. FAK, expressed in 48–75% of pancreatic cancers, is a non-receptor cytoplasmic tyrosine kinase involved in the regulation of cellular signalling, migration, apoptosis and cell cycle progression [124–127]. It is also associated with invasive potential. Gene silencing with RNAi promoted anoikis and inhibited metastasis of human pancreatic cancer in an animal model, as well as enhancing gemcitabine chemosensitivity [126].

Smad4 and TGF-β

Mutation or deletion of the Smad4 (MADH4) gene, originally designated as the tumour suppressor gene DPC4 (deleted in pancreatic carcinoma, locus 4) on chromosome 18q21.1, occurs in 55% of pancreatic cancers [128]. It is a member of the Smad family of transcription factors, and Smad4 inactivation potentiates tumour growth, angiogenesis [129] and invasion [130, 131] and is associated with poor prognosis [132]. Formation of a heteromeric complex between TGF-β ligand and TGF-β type I and type II receptors (TβRI and TβRII) leads to phosphorylation of cytoplasmic Smad2 and Smad3 proteins by TβRI kinase, which in turn form heteromeric complexes with Smad4 [133, 134]. Restoration of the Smad4 gene using an adenoviral vector showed inhibition of pancreatic tumour growth in mice, although oddly a significant effect on proliferation was not seen in vitro [131]. Suppression of tumour growth was mediated in part by the downregulation of VEGF and expression of gelatinases (involved in tumour growth, invasion, angiogenesis and promotion).

TGF-β plays a complex role as it is tumour suppressive in epithelial cells, but can also promote invasion and metastasis during the later stages of carcinoma progression [135]. Mutations of TβRI and TβRII are found in 1 and 4% of pancreatic cancers, respectively [136]. Knockdown of Smad4 resulted in TGF-β-induced cell cycle arrest and migration but not in TGF-β-induced epithelial-mesenchymal transition, which makes cells more migratory and invasive. TGF-β-based therapeutic strategies in can-

cer are in development [135]. The antisense oligonucleotide specific for human TGF-β2 mRNA, AP 12009, was initially tested for use in high-grade glioma [137, 138]. In laboratory studies, AP 12009 significantly reduced TGF-β2 secretion in human pancreatic cancer cell lines (Hup-T3, Hup-T4, PaTu-8902), decreased proliferation by up to 76%, blocked tumour migration, as well as reversed TGF-β2-mediated immune suppression in which lymphokine-activated killer cell cytotoxicity was increased up to 708% [139]. Ongoing phase I/II studies for the treatment of pancreatic carcinoma, malignant melanoma and colorectal carcinoma have been reported to show promising results, with one advanced pancreatic cancer patient still alive 72 weeks after having experienced a complete response [140].

Hedgehog Signalling

Hedgehog (Hh) signalling specifies the pattern and structure of many tissues during embryonic development. There are three mammalian Hh proteins, namely Sonic (Shh), Indian (Ihh) and Desert Hh (Dhh), respectively. Activation of the Hh pathway is controlled by two transmembrane proteins, the tumour suppressor Patched (Ptc) or the oncogenic Smoothened (Smo) [141]. Ptc normally suppresses Smo, but binding of Hh to Ptc relieves this inhibition, leading to Smo activation of transcriptional response [142]. Shh is expressed in 70% of human pancreatic adenocarcinomas [143]. Although also found in premalignant lesions, it is not detectable in normal ductal epithelium. Ihh expression is increased 35-fold in pancreatic cancer compared to normal tissues [144]. Mechanisms of tumourigenesis include its effects on the cell cycle regulators cyclin D1 and p21, protection from apoptosis via the PI3K/Akt signalling and stabilisation of Bcl-2 and Bcl-xL, as well as its collaboration with activated K-ras [145, 146].

Cyclopamine binds to Smo and inactivates the Hh pathway. It inhibited cell growth in vitro and in vivo for a wide range of digestive tract tumours, including the pancreas [147]. Killing of pancreatic cancer cells in vitro by paclitaxel or radiation was enhanced by cyclopamine, but this was not seen with gemcitabine or cisplatin [148]. In an orthotopic pancreatic xenograft model, only 1 of 7 cyclopamine-treated mice developed pulmonary micrometastases compared to controls in which all developed multiple macrometastases [149]. Addition of gemcitabine completely prevented metastasis whilst inhibiting tumour growth at the primary site. Cyclopamine also downregulates the expression of EGFR, and in vitro studies of this agent in combination with gefitinib showed that it profoundly reduced the growth of Panc-1, SUIT-2 and AsPC-1 cells [150].

GLI-1 is a transcription factor that mediates the Shh pathway. Its inhibition by a synthetic micro RNA (miRNA-GLI-1-3548) was reported to suppress proliferation and induce apoptosis in MIAPaCa-2 cells [151].

Notch Signalling

Notch signalling is important in the development of organs, affecting tissue proliferation, differentiation and apoptosis. There are four known Notch genes in mammals that encode for heterodimeric transmembrane receptors. Its ligands are from two families of proteins known as 'Delta' and 'Jagged', respectively. Activation leads to proteolytic cleavage of the Notch receptors by γ-secretase, releasing the cytoplasmic domain which migrates to the nucleus and binds to transcription factors such as CSL (CBF-1 in mammals, suppressor of hairless in *Drosophila* and LAG-1 in *Caenorhabditis elegans*).

Notch signalling has been shown to be a downstream event of ras, EGFR and TGF-α in pancreatic tumourigenesis [152, 153]. It also promotes tumour neovascularisation [154]. Downregulation of Notch1 with siRNA inhibited cell growth and induced apoptosis in BxPC-3, HPAC and Panc-1 pancreatic cancer cells [155]. This also resulted in the reduction of NFκB, VEGF and MMP-9 with subsequent inhibition of cell invasion [156], as well as enhanced antitumour activity in combination with curcumin via NFκB inhibition [157]. Notch3 is found in around 70% of pancreatic cancers and is associated with a more aggressive tumour phenotype [158, 159]. Inhibition by siRNA downregulated Bcl-xL in BxPC-3 cells, whilst γ-secretase inhibitors (GSI and L-685,458) resulted in decreased proliferation of Panc-1, HPAF-2 and BxPC-3 cells [159].

Oncogenes and Tumour Suppressor Genes

Cancer-Associated Sm-Like Protein

Cancer-associated Sm-like protein (CaSm), also known as human Sm-like protein (LSM1, hLsm1), is overexpressed in the majority of pancreatic cancers and encodes a 1.2-kb mRNA transcript, with the largest open reading frame encoding a 133-amino-acid protein that contains two Sm motifs found in the common small nuclear RNA proteins and the LSm family of proteins [160, 161]. LSm family of proteins are involved in mRNA decapping and degradation [162]. Antisense CaSm RNA was able to alter the transformed phenotype of pancre-

atic cancer cells by reducing their ability to form large colonies in soft agar [160]. An adenoviral vector engineered to express CaSm antisense RNA (Ad-αCaSm) significantly inhibited tumour growth both in vitro and in vivo primarily by disrupting cell cycle progression, and the antitumour effect was further enhanced by gemcitabine [163]. Systemic administration of Ad-αCaSm also resulted in a reduction of the number of hepatic metastases and an increased survival time of mice bearing the murine pancreatic cell line Panc02 [164]. The antitumoral efficacy was dependent on both direct and bystander mechanisms.

BIRC5 (Survivin)

Survivin is a 16.5-kDa protein encoded by a gene known as BIRC5 (baculoviral inhibitor of apoptosis repeat-containing 5) on the telomeric position of chromosome 17. It functions as an antiapoptotic and cell cycle regulatory protein by blocking the common downstream effectors of both the intrinsic mitochondrial and the extrinsic membrane death receptor pathways, namely caspases-3, -7 and -9 [165]. Survivin is expressed in more than 80% of pancreatic cancers, some premalignant lesions but not in nonneoplastic pancreatic tissues [166–168], and is associated with poor clinical outcome [168–170].

When pancreatic cancer cell lines were irradiated, survivin mRNA expression was upregulated to induce increased radioresistance [171]. siRNA treatment could improve the radiosensitivity of AsPC-1 cells [172, 173]. It also inhibited growth and induced apoptosis in Panc-1 and PC-2 cells [173, 174]. The survivin antisense oligonucleotide (LY2181308) was able to produce significant antitumour activity in human xenograft tumour model in mice [175].

$p16^{INK4A}$/$p21^{CIP1/WAF1}$/Retinoblastoma Protein

These are tumour suppressor genes that regulate the G1 to S checkpoint of the cell cycle [176]. In response to mitogenic signals, cyclin D is upregulated which in turn activates cyclin D-dependent kinases 4 and 6 (CDK4 and 6). This leads to the phosphorylation of pRb, resulting in the release of transcription factor E2F, therefore inducing the expression of genes needed for DNA synthesis. pRb can also be phosphorylated by cyclin E-dependent kinase 2 (CDK2) in late G1 phase. $p16^{INK4A}$ binds and inactivates CDK4/6, whilst $p21^{CIP1/WAF1}$, induced by p53, inhibits CDK2.

The $p16^{INK4A}$ (MTS1) gene on chromosome 9p21, is deleted in 85% of pancreatic adenocarcinomas [177]. p16-mediated cytotoxicity is tightly associated with the presence of functional pRb [178]. This is advantageous for gene therapy in pancreatic cancer as only 6% showed mutant pRb [179]. E1- and E3-deleted adenovirus 5 containing p16 (AdexCACSp16) induced a high level of p16 mRNA expression in MIAPaCa-2 cells, significantly suppressing cell proliferation [180]. When infected with a replication-defective adenovirus containing the $p21^{CIP1/WAF1}$ gene (rAd-p21), HPAC and Hs766T cells showed significant growth inhibition in vitro [181].

p53

The tumour suppressor gene p53, located on chromosome 17p, is inactivated by mutation in 50–75% of pancreatic adenocarcinomas [182]. It encodes a transcription factor that is upregulated and activated upon stress such as DNA damage [176]. It can induce apoptosis or G1 cell cycle arrest via $p21^{CIP1/WAF1}$. It is normally maintained at a very low level by mdm2 (murine double minute) which targets p53 for ubiquitin-mediated degradation. Stress or mitogenic signals increase the level of $p14^{ARF}$ which in turn inhibits mdm2, leading to the stabilisation and activation of p53. Recently it was discovered that the DNA damage-induced p53 response is dispensable for tumour suppression, but instead $p19^{ARF}$, induced by oncogenic disruption of the cell cycle, plays a crucial role [183].

The first gene therapy for the treatment of cancer was approved in China in 2004, where Gendicine (SiBiono GeneTech, Shenzen, China), a replication-defective adenovirus 5 expressing p53, is used for squamous cell carcinoma of the head and neck [184]. In pancreatic cancer, transfer of p53 using a similar vector (Ad5/CMV/p53) suppressed the growth of human pancreatic cancer cell lines AsPC-1, BxPC-3, Capan-1, CFPAC-1, MIAPaCa-2 and Panc-1. Suppression of tumour growth mediated by apoptosis was observed in nude mouse subcutaneous tumour model [185]. A retroviral p53 vector inhibited growth of primary as well as peritoneal deposits of BxPC-3 in mice [186]. Reintroduction of p53 using adenoviral vector to cells previously treated with gemcitabine increased cytotoxicity both in vitro and in vivo, although this effect was not seen with cisplatin [187].

Thoc1/p84 (also known as hHpr1 or p84N5), a protein that localises in the subnuclear regions associated with RNA processing, binds to pRb [188]. A recent study found that infection of pancreatic adenocarcinoma with adenovirus encoding p53 and Thoc1/p84 inhibited growth in vitro and in vivo to a greater extent than treatment with either one alone [189].

p73

p73, localised on chromosome 1p36, is a proapoptotic gene of the p53 gene family observed in 45.6% of pancreatic adenocarcinomas [190]. Overexpression of p73 is inversely linked to lymph node metastasis and tumour size. It can induce cell cycle arrest and apoptosis in a p53 manner. An adenoviral vector that encodes p73 was capable of effective killing of several pancreatic cancer cell lines, including those that were completely resistant to p53-mediated apoptosis [191].

Suicide Gene Therapy

Also called gene-directed prodrug activation therapy, suicide gene therapy is a two-step process. First a gene is delivered to the tumour that will lead to the expression of an enzyme. A prodrug is subsequently administered that is activated selectively by this enzyme.

Herpes Simplex Virus Thymidine Kinase/Ganciclovir

The herpes simplex virus thymidine kinase (HSV-tk)/ganciclovir approach is the most well-known example of suicide gene therapy. HSV-tk is able to monophosphorylate the guanosine analogue ganciclovir, which is subsequently converted by cellular guanylate kinases to the triphosphorylated forms, blocking DNA synthesis and inducing cell death [192]. The therapeutic effect is also based on a 'bystander effect' whereby HSV-tk-transduced tumour cells are toxic to nearby neighbouring unmodified tumour cells. This method, delivered by a retroviral vector, seemed to be effective in inhibiting the growth of the human pancreatic cancer cell line SW1990 in vitro [193]. Similar results were also found both in vitro and in vivo for DSL-6A/C1 rat pancreatic carcinoma cells with HSV-tk transduced retrovirally and adenovirally [194], as well as in nude mice bearing human pancreatic cancer cells intraperitoneally after treatment with Ad-HSV-tk [195]. Murine pancreatic tumour, injected into the liver, displayed significant tumour volume reduction and necrosis after intratumoural injection of HSV-tk-bearing adenoviruses followed by intraperitoneal ganciclovir administration [196]. An in vivo study showed that the combination of adenovirus- and retrovirus-mediated delivery of HSV-tk appeared to be more effective in tumour reduction compared to either one alone [197]. Liposome-mediated transfer of HSV-tk caused regression of tumours in nude mice with peritoneal dissemination of the human pancreatic cancer cells PSN-1 [198]. Other studies, however, indicated that retrovirally transduced HSV-tk has limited efficacy in human pancreatic cell lines both in vitro and in vivo [199, 200].

Cytosine Deaminase

Cytosine deaminase is a bacterial enzyme that converts the prodrug 5-fluorocytosine (5-FC) into the cytotoxic and radiosensitising agent 5-FU. A replication-defective adenovirus carrying the CD gene was found to inhibit the growth of the murine pancreatic cancer cell line Panc02 both in vitro and in vivo when given together with 5-FC [201]. Similar results were also demonstrated for PaTu-8988 and SW1990 cells in vitro [202, 203]. A phase I trial of this virus, injected intratumourally under ultrasound guidance, is being tested with chemoradiotherapy in patients with non-metastatic pancreatic adenocarcinoma. A retrovirus with the CD gene linked to the oncogene ErbB2 promoter enhanced cell killing with 5-FC in ErbB2-positive pancreatic cancer cells [204].

FCY1 and FUR1 are genes encoding for CD and uracil phosphoribosyltransferase (UPRT), respectively. They are derived from the yeast *Saccharomyces cerevisiae*. As some cells are relatively resistant to 5-FU, UPRT has an additional advantage as it catalyses the conversion of 5-FU into the toxic metabolite 5-fluorouridine-5′-monophosphate [205]. The results of a recent in vitro study of these genes, transfected using plasmid vectors into human pancreatic cancer cell lines, were however far from impressive [206]. FCY1 alone was ineffective. In combination with FUR1, only some showed increased sensitivity to 5-FC. In another study of an E1B-55-kDa-deleted adenovirus carrying the UPRT gene (AxE1AdB-UPRT) together with 5-FU, mice with peritoneal dissemination of AsPC-1 showed dramatic improvement without toxicity to normal tissues [207].

Nitroreductase

The *Escherichia coli* enzyme nitroreductase (NTR) is able to reduce the prodrug CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] to 2- and 4-hydroxylamino derivatives, the latter then reacts with cellular thioesters to generate a potent alkylating agent capable of cross-linking DNA [208]. In contrast to HSV-tk/ganciclovir and CD that work by inhibiting DNA synthesis, NTR/CB1954 is also toxic to non-replicating tumour cells. Pancreatic cancer cell lines (SUIT-2, BxPC-3 and AsPC-1) expressing the NTR gene (introduced retrovirally) showed increased sensitivity to CB1954 (up to 500-fold in SUIT-2), with associated bystander effect [209]. In nude mice bearing subcutaneous NTR-expressing human pancreatic cancer cells, CB1954 administration resulted in tumour

regression, growth delay and increased MS [210]. In a murine xenograft model with peritoneal dissemination of SUIT-2, administration of a replication-defective adenoviral vector containing the NTR gene (Ad-CMV-ntr) in combination with CB1954 almost doubled MS from 14 to 26 days [211]. No clinical trial has yet been done in patients with pancreatic cancer.

Antiangiogenic and MMP Inhibitors

Antiangiogenic Inhibitors

Tumour growth is dependent on angiogenesis, a process involving the VEGF family of proteins and receptors. VEGF is a glycoprotein that promotes endothelial cell survival, mitogenesis, migration, differentiation, and vascular permeability [212]. It is overexpressed in over 90% of pancreatic cancers and is associated with increased microvessel density, tumour progression and poor prognosis [213]. The VEGF receptors, VEGFR-1 (FMS-like tyrosine kinase-1 – Flt-1) and VEGFR-2 (foetal liver kinase-1 – Flk-1, or kinase insert domain receptor) are also overexpressed in the vasculature of tumours that express VEGF [214]. Growth factors such as EGF, TGF-α, TGF-β, platelet-derived growth factor (PDGF), and the cytokines IL-1α and IL-6, can upregulate the expression of VEGF [215].

Soluble VEGFR-2

Soluble forms of VEGFR-1 and VEGFR-2 could inhibit VEGF-dependent tumour angiogenesis, first by sequestering VEGF, and secondly by forming heteromeric complex with their wild-type receptor thus acting as a dominant negative [216]. A recombinant adenovirus encoding a soluble form of VEGFR-2 (Ad Flk1-Fc) showed significant antitumour effect when injected intravenously into mice bearing either Panc02 or BxPC-3 cancer cells [217]. A truncated dominant negative mutant of VEGFR-2, when delivered via replication-defective retroviruses, resulted in inhibition of tumour growth in each of three human pancreatic cancer cell lines in vivo [218].

AS-3

AS-3 is a VEGF antisense oligonucleotide that has been tested in mice with the human pancreatic cancer cell lines AsPC-1 or HPAF-2 implanted into the pancreas [219]. AS-3 normalised plasma VEGF level, decreased angiogenesis, reduced tumour growth and metastasis with subsequent improved survival. None of the treated animals developed ascites, suggesting a reduction in vascular permeability caused by decreased VEGF.

PTK 787 (ZK222584) and SU5416

Blockade by the VEGF tyrosine kinase inhibitor PTK 787 has been shown to hinder the growth of L3.6pl pancreatic cancer xenograft by inhibiting angiogenesis, cell proliferation and increased apoptosis. When combined with gemcitabine, there was significant reduction in lymph node and hepatic metastases and increase in survival [220, 221]. SU5416, a selective inhibitor of VEGFR-2 tyrosine kinase, produced a significant antitumour effect in vivo on MIAPaCa-2 cells, with an almost complete suppression of tumour growth and relapse when combined with gemcitabine [222].

ZD6474

ZD6474 is an orally active inhibitor of VEGFR-2 and EGFR tyrosine kinase. This was tested on mice bearing L3.6pl cancer cells [223]. ZD6474 decreased primary tumour growth, lymph node and hepatic metastases compared to treatment with gemcitabine alone. Antitumour effect was more marked when ZD6474 was given in combination with gemcitabine. ZD6474 has also been tested with ionising radiation and gemcitabine, both in vitro to MIAPaCa-2, Panc-1 and Capan-1 cells, and in vivo to MIAPaCa-2 tumour xenografts, with encouraging results [224].

Axitinib (AG-013736)

Axitinib is an orally active inhibitor of VEGFRs, as well as related tyrosine kinase receptors at higher concentration, namely PDGF receptor-β (PDGFR-β) and c-Kit [225]. In vivo studies have demonstrated that it has potent anti-angiogenic and anti-tumour activities [226–228]. In a recent phase II trial of 103 patients with advanced pancreatic cancer, the reported MS was 6.9 months when axitinib was given in combination with gemcitabine, compared to 5.6 months with gemcitabine alone [229]. It is currently in phase III trial.

Sorafenib (BAY 43-9006)

Sorafenib has an inhibitory effect on VEGF, PDGFR-β, c-Kit, raf-1 and Flt-3 that are important for tumour proliferation and angiogenesis [230]. It has been approved by the US Food and Drug Administration in 2005 for the treatment of advanced renal cell carcinoma. A phase II trial showed that although it was well tolerated, it was inactive in patients with advanced pancreatic cancer [231]. A phase III trial is in progress.

NK4 and ARQ 197

NK4 (natural killer transcript 4) is a synthetic competitive antagonist of hepatocyte growth factor (HGF) and an angiogenesis inhibitor [232]. HGF binds to the c-Met-encoded receptor, which is overexpressed in 61–87% of pancreatic cancers [233–235]. HGF is infrequently expressed by pancreatic cancer cells, but tumour-associated fibroblasts do produce HGF [230, 236, 237]. HGF promotes growth, enhances cell motility and extracellular matrix breakdown leading to invasion and metastasis of cancer cells. In pancreatic cancer mouse tumour model, NK4 suppressed tumour progression by inhibiting both angiogenesis and HGF-mediated invasion/metastasis [236], although the latter mechanism has been debated [238].

An NK4-expressing adenoviral vector (Ad-NK4) has been tested on pancreatic cancer cells both in vitro and in vivo. Ad-NK4 potently inhibited invasion of cancer cells in response to HGF [239]. Intrasplenic injection of Ad-NK4 suppressed the number and growth of hepatic metastases [240]. Intraperitoneal injection of Ad-NK4 suppressed the development of AsPC-1 tumour in a mouse peritoneal dissemination model [241]. In mice with orthotopically implanted SUIT-2 tumours, peritumoural injection of Ad-NK4 in combination with gemcitabine significantly reduced tumour volume compared to Ad-NK4 or gemcitabine alone [242]. Complete suppression of peritoneal dissemination and liver metastases was also noted, leading to improved survival of the animals.

ARQ 197, a c-Met receptor tyrosine kinase inhibitor, is currently in phase II trial. Phase I study showed that it was well tolerated, and of the 33 evaluable patients, two achieved partial response and 19 had stable disease [243].

TNP-470

TNP-470 [O-(chloroacetyl-carbamoyl) fumagillol] is an analogue of fumagillin derived from *Aspergillus fumigatus* that could inhibit the proliferation of endothelial cells [244]. Early studies on pancreatic cancer have shown that it is effective in inhibiting hepatic metastasis of human pancreatic cancer cells in mice following splenic injection, with or without cisplatin [245, 246]. Significant inhibition of tumour growth, metastatic spread and angiogenesis has also been demonstrated in vivo for MIAPaCa-2, AsPC-1 and Capan-1, although survival was not statistically significant [247]. TNP-470 was tested with gemcitabine in nude mice with surgically implanted SW1990 cells in the tail of the pancreas [248]. When used alone, gemcitabine inhibited the growth of the primary tumour more significantly than TNP-470 alone, whereas tumour metastasis was better controlled with TNP-470. No significant improvement in survival rate was noted in these two groups. However, when administered in combination, there were improved anti-tumour and anti-metastatic effects as well as increased survival.

Cilengitide (EMD 121974)

Integrins are heterodimer transmembrane receptors composed of α- and β-subunits responsible for cell adhesion to the extracellular matrix. They bind to ligands that include laminin, fibronectin, vitronectin, thrombospondin, fibrinogen and fibrin. Integrins play a role in angiogenesis where they are involved in endothelial cell migration, proliferation and survival [249]. αVβ3 induces angiogenesis via basic fibroblast growth factor or tumour necrosis factor-α (TNF-α), whereas αVβ5 achieves this via VEGF or TGF-α [250].

Cilengitide is an inhibitor of the integrins αVβ3 and αVβ5 [251]. In a phase II study of 89 patients, no significant response was noted [252]. The MS for cilengitide with gemcitabine was 6.7 compared to 7.7 months for gemcitabine alone, with overall response rates of 17 and 14%, respectively.

MMP Inhibitors

The interplay between pancreatic cancer cells and its surrounding tumour stroma plays an important role in tumour progression, affecting growth, angiogenesis, evasion of host immune response, invasion and metastasis [253]. Degradation of the extracellular matrix by MMPs, a family of zinc-containing proteolytic enzymes, is essential for tumour spread and neovascularisation. The imbalance between activated MMPs and tissue inhibitors of MMPs is frequently found in pancreatic cancer [254].

Despite evidence on the effectiveness of various MMP inhibitors (MMPIs) in animal models [255–259], results of clinical trials have been disappointing. Marimastat is a synthetic broad-spectrum, orally active MMPI. Although well tolerated (musculoskeletal toxicity is the most severe side effect), it did not show any survival benefit for patients with advanced pancreatic cancer compared to gemcitabine or in combination [260, 261]. Another MMPI, BAY 12-9566, was inferior to gemcitabine in a phase III trial [262].

Oncolytic Virus

Replication-selective oncolytic viruses are engineered to replicate specifically in and destroy tumour cells. Viruses kill cells by a number of mechanisms which include direct cell lysis, expression of toxic viral proteins, induction of antitumour immunity, and sensitisation to their effects. The first evidence of efficacy of oncolytic viral therapy was described in 1912 by DePace, when a patient with uterine cervical carcinoma experienced tumour regression after an attenuated rabies vaccination. Since then numerous types of oncolytic viruses have been evaluated as therapeutic agents, most notably adenoviruses [263].

ONYX-015

Adenovirus is a non-enveloped, icosahedral, double-stranded DNA virus of about 70–90 nm in diameter, with a genome size of 34–48 kb. ONYX-015 (dl1520) is a replication-selective adenovirus type 5 with E1B-55-kDa gene deletion. Normally the E1B-55 kDa protein is able to bind and inactivate p53, an essential step for effective viral replication [264]. It is thought that as most tumours have lost the function of the p53 pathway, deletion of E1B-55 kDa would enable ONYX-015 to selectively replicate in cancer cells but not in normal cells.

Promising laboratory results have led ONYX-015 to be the first replication-selective oncolytic virus to enter clinical trials and the world's first approved for head and neck cancer therapy. In a phase I trial, ONYX-015 was administered via CT-guided (22 patients) or intraoperative injection (1 patient) into pancreatic primary tumours every 4 weeks until tumour progression [265]. Six patients showed 25–49% tumour regression, 11 were stable, and five showed tumour progression. A phase I/II study of 21 patients was done to evaluate the use of endoscopic ultrasound-guided intratumoural injection of advanced pancreatic carcinomas with ONYX-015 and then in combination with systemic gemcitabine [266]. Two had partial progression, 2 had minor reponse, 6 had stable disease, and 11 progressed or had to go off the study because of treatment toxicity. Complications included infection and duodenal perforation from the rigid endoscope tip. Disappointingly in these trials, viral replication was not detectable on fine needle biopsy of the tumours, unlike other trials for head and neck cancers [267, 268], liver metastases of colorectal carcinoma [269], and ovarian cancer [270].

However, it is important to note that the interaction between E1B-55 kDa and p53 is more complex than originally thought, because ONYX-015 could replicate in some tumour cells that retain the wild-type p53 [271]. It was later shown that tumour selectivity of ONYX-015 is determined not by p53, but by the export of late viral RNA, a function requiring E1B-55 kDa in normal but not in tumour cells [272].

Herpes Simplex Virus

HSV is a large, enveloped, double-stranded DNA virus with a genome size of approximately 152 kb. There are two serotypes of HSV, namely HSV-1 and HSV-2.

G207 is a replication-selective mutant of HSV-1 with deletions at both γ34.5 loci and a lacZ reporter gene inserted in and thus disrupting the UL39 gene [273]. γ34.5 prevents the shut-off of host protein synthesis in infected cells by interacting with cellular phosphatase-1α to dephosphorylate eIF-2α, leading to the production of more progeny viruses from infected cells [264]. UL39 normally encodes for the infected cell protein 6, the large subunit of ribonucleotide reductase required in the biosynthesis of DNA. Given their functions, γ34.5- and UL39-deleted mutants are unable to replicate in normal cells but they can do so in actively dividing tumour cells. NV1020 is derived from HSV-1 and contains deletions in the endogenous thymidine kinase gene (HSV-tk) and in one of the two γ34.5 genes. HSV-tk is normally needed for nucleic acid metabolism, therefore mutant virus is dependent on endogenous levels of this enzyme, which is found in high levels in replicating cells [264]. NV1020, however, does contain an exogenous copy of the HSV-tk gene derived from HSV-2, thus maintaining its sensitivity to aciclovir and ganciclovir which would otherwise be lost with the disrupted HSV-tk gene. In vitro laboratory findings showed that G207 can replicate in and kill human pancreatic cell lines AsPC-1, MIAPaCa-2 and BxPC-3 [274]. In a separate study, both G207 and NV1020 were equally effective in lysing four human pancreatic cells in vitro [275]. For Hs766T flank tumours in athymic mice, tumour eradication was achieved in 25% of the animals with G207 and 40% with NV1020, respectively.

OncoVEX$^{GM\text{-}CSF}$ is a recombinant HSV-1 with γ34.5 and ICP47 gene deletions, together with the human granulocyte-macrophage colony-stimulating factor (GM-CSF) gene inserted. ICP47 normally binds to the transporter associated with antigen processing to prevent

the delivery of peptides into the endoplasmic reticulum, where they bind to major histocompatibility complex class I molecules. The genetic alterations of OncoVEX$^{GM\text{-}CSF}$ mean that it could replicate selectively in tumour cells whilst boosting the antitumour immune response. It will be tested in a phase I trial in patients with unresectable pancreatic cancer.

hrR3 (UL39 deleted, HSV-tk conserved) was tested on nude mice bearing peritoneal dissemination of the human pancreatic cancer cells SW1990 [276]. Long-term survival was seen in 70% of mice receiving hrR3 and ganciclovir, 40% receiving hrR3 alone, and none of the untreated animals. In another study, hrR3 and R3616 (γ34.5 deleted) were given with gemcitabine to pancreatic cancer cell lines [277]. There was more cytotoxicity when each of the viruses was given in combination with gemcitabine in vitro, although viral replication was inhibited. In a murine model with peritoneal dissemination, R3616 with gemcitabine had a greater effect than R3616 alone, while hrR3 with gemcitabine had a weaker effect than hrR3 alone.

FusOn-H2, a mutant HSV-2 with deletion of the protein kinase (PK) domain of the viral ICP10 gene, has shown promising in vivo results for pancreatic cancer [278]. The PK domain is normally required for HSV-2 replication, where it binds and phosphorylates ras, leading to activation of the ras/MAPK/ERK pathway, as well as the expression and stabilisation of the transcription factor c-Fos [279]. FusOn-H2 can therefore replicate selectively in pancreatic cancer cells in which the majority have an activated ras signalling pathway. In mice bearing subcutaneous MPanc-96 tumours, a single intratumoural injection of FusOn-H2 completely eradicated the disease, whilst intraperitoneal injection cleared established orthotopic tumours in 75% of the mice and completely prevented local metastasis.

The US3 gene encodes a serine/threonine PK that protects cells from apoptosis [280]. L1BR1 is a US3 locus-deficient HSV-2 mutant. When tested on mice bearing SW1990 tumours, L1BR1 showed significantly better antitumour effect compared to hrR3 and R3616 (γ34.5 double-deletion mutant) [281]. It also showed the lowest replication capacity in normal human hepatocytes, and enhanced tumour apoptosis in vitro (SW1990, Capan-2 and BxPC-3) in combination with cisplatin and 5-FU.

The agent d120.surv is a recombinant HSV-1 that contains a survivin promoter driving the expression of ICP4, a major transactivating factor for viral genes. This makes replication of the virus restricted to survivin-expressing cancer cells. In vitro cytotoxicity was significantly higher in AsPC-1 (high survivin expression) compared to Panc-1 (low survivin expression) [282].

Reovirus

Reovirus is a non-enveloped, double-stranded RNA virus that normally causes subclinical infection in humans. Replication of reovirus requires an activated ras signalling pathway [283]. Normally when reovirus infects a cell, the intracellular host defence system is activated by the RNA-activated PK (PKR). This leads to PKR autophosphorylation, followed by phosphorylation of eIF-2α, which in turn inhibits protein synthesis, thus preventing viral replication. However, in cells with activated ras, PKR phosphorylation is blocked.

Reovirus was able to infect human pancreatic cancer cells Panc-1, MIAPaCa-2, PK-1, PK-9 and BxPC-3 in vitro [284]. In nude mice bearing Panc-1 and BxPC-3, tumour growth was inhibited by intratumoural injection, whilst local injection also had systemic antitumour effect in a bilateral xenograft model. Immunohistochemical staining showed viral replication in tumour but not in the surrounding normal tissue. In a separate study of pancreatic hepatic metastases in hamster, intraportal administration of reovirus significantly reduced the size and number of tumours [285].

Immunotherapy

In the first attempt of cancer immunotherapy, William Coley in the 1890s injected bacterial components into the tumours of his patients, activating the immune system which contributed to tumour cell rejection. In 1967 Lindenmann and Klein discovered that vaccination of mice with influenza virus-infected tumour cells showed antitumour response to non-infected parental cells, suggesting that the immunogenicity of host cell components was greatly increased by incorporation into the makeup of the virus [286]. Compelling evidence now suggests that the immune system plays an important role in the control of malignancy [287], and immunotherapy is widely considered as the most important and rapidly growing area of cancer research. Passive immunotherapy includes the use of antitumour agents that have been generated in vitro, such as the use of antibodies or effector cells, whereas active immunotherapy aims to stimulate an antitumour response in vivo by means of vaccination.

Antibodies

Anti-EGFR Antibodies

Cetuximab (Erbitux or IMC-C225) is a chimeric monoclonal antibody generated from fusion of the variable region of the murine anti-EGFR monoclonal antibody M225 and the human IgG_1 constant region. Promising laboratory results have led cetuximab to be tested in clinical trials. However, a recent phase III trial of 766 patients with locally advanced or metastatic pancreatic adenocarcinoma failed to demonstrate any significant benefit (MS of 6 months with gemcitabine vs. 6.5 months in combination with cetuximab, p = 0.14) [288]. The corresponding PFS was 3 and 3.5 months, respectively (p = 0.058). In an ongoing phase II trial with trimodal therapy of cetuximab, gemcitabine and intensity-modulated radiotherapy for patients with locally advanced pancreatic cancer, there was no increase in toxicity profile [289]. One-year survival was 57% whilst MS has not been reached. Of the 36 patients 12 showed partial remission, 20 had stable disease, and 4 had progressive disease. Combination of cetuximab, gemcitabine and radiotherapy caused the most growth inhibition on MIAPaCa-2 and BxPC-3 cells when compared to single or double combination therapy [290].

Matuzumab (EMD72000) is a humanised IgG_1 monoclonal antibody to the human EGFR. Laboratory studies have shown promising inhibitory effects on tumour growth and angiogenesis, including L3.6pl in an orthotopic rat model [291]. In a phase I study of combined treatment with matuzumab and gemcitabine, 8 out of 12 patients with advanced pancreatic adenocarcinoma showed partial response or stable disease [292]. It appeared to be well tolerated.

Anti-VEGF Antibody

Bevacizumab (Avastin) is a recombinant humanised anti-VEGF monoclonal antibody. Despite promising phase II results [293], the recent phase III CALGB 80303 trial did not show any survival benefit of bevacizumab/gemcitabine combination compared to gemcitabine alone in 602 patients with advanced pancreatic cancer (MS of 5.7 vs. 6 months) [294]. The AviTa phase III trial that examined treatment with gemcitabine plus erlotinib with either bevacizumab or placebo has been closed. Bevacizumab, however, may have a role in palliative treatment of chemotherapy-resistant pancreatic cancer. In a case report, a patient with stage IV disease initially unresponsive to gemcitabine, 5-FU, irinotecan and cisplatin subsequently responded with the addition of bevacizumab [295].

Anti-MUC1 Antibodies

MUC1 (mucin-1, CD227) is a polymorphic, glycosylated type I transmembrane protein expressed on glandular epithelium including the pancreas, breast, lung and gastrointestinal tract. It is overexpressed in 90% of pancreatic cancers and aberrantly glycosylated [296, 297]. It inhibits cell-cell and cell-stroma interactions and functions as a signal transducer in the progression of cancer, including tumour invasion and metastasis [298]. Evidence suggests that circulating anti-MUC1-IgG antibodies are a favourable prognostic factor for pancreatic cancer [299]. Down-regulation of MUC1 expression in S2-013 human pancreatic cancer cell line by RNAi significantly decreased proliferation in vitro and in nude mice [300]. Adhesion of MUC1-suppressed cells to type IV collagen and fibronectin was slightly increased, and adhesion was slightly decreased to type I collagen and laminin. Implantation of these cells into the caecum or pancreas showed significant reduction of lymph node, pulmonary and peritoneal metastases. Similarly, siRNA towards MUC1 reduced cell proliferation and enhanced sensitivity to genotoxic drugs in MIAPaCa-2 and Capan-1 cells, whilst the overexpression of MUC1 in the normally low MUC1-expressing BxPC-3 cells caused high proliferation with low basal apoptosis [301].

^{90}Yttrium-labelled PAM4 monoclonal antibody that recognises MUC1 was studied in combination with gemcitabine as a radiosensitiser in mice bearing Capan-1 [302]. There was increased inhibition of tumour growth and prolonged survival of the animals. It is currently undergoing phase I trial for patients with stage III or IV pancreatic cancer. ^{213}Bi-C595 is an antibody targeting the protein core of MUC1, conjugated with the α-particle-emitting ^{213}bismuth. In vitro study showed that ^{213}Bi-C595 was specifically cytotoxic to MUC1-expressing pancreatic cancer cells in a concentration-dependent manner compared to controls [297].

Anti-Mesothelin Antibodies

Mesothelin is a 40-kDa protein present on normal mesothelial cells of the pericardium, pleura and peritoneum, but is overexpressed in mesotheliomas, pancreatic and ovarian cancers [303]. Detected in 90–100% of pancreatic adenocarcinomas [304, 305], diagnosis by fine-needle aspiration biopsies using mesothelin as a marker has a sensitivity of 68% and specificity of 90% [306].

SS1P is a recombinant immunotoxin that consists of an anti-mesothelin Fv (SS1) fused to PE38, a 38-kDa portion of *Pseudomonas* exotoxin A. After binding to mesothelin and subsequent internalisation into cells, it inhib-

its protein synthesis and results in apoptosis. A phase I study showed that it was well tolerated with self-limiting pleuritis as the dose-limiting toxicity, due to the binding of SS1P to normal mesothelial cells [307].

MORAb-009, a monoclonal antibody against mesothelin, is being tested in a phase I trial of 11 patients (3 had pancreatic cancer) [308]. One of them who had previously progressed on gemcitabine showed disease stabilisation on CT and a drop in CA19-9.

Anti-Ptc Antibody

Antibody against Ptc has been shown to suppress Hh signalling and proliferation of the human pancreatic cells Panc-1 and SUIT-2 [309].

RAV12

RAV12 is a chimeric IgG_1 antibody against RAAG12, an N-linked carbohydrate epitope expressed in up to 100% of pancreatic adenocarcinomas, as well as the majority of gastric and colorectal cancers [310]. Intraperitoneal injection of RAV12 inhibited the growth of SU 86.86 pancreatic cancer cells in a subrenal capsule xenograft mouse model [310]. Preliminary data from an ongoing phase I study of patients with recurrent adenocarcinoma revealed that of the 4 patients with pancreatic cancer, one with advanced disease had a >50% reduction in CA19-9 [311].

Adoptive Cell Transfer

Adoptive immunotherapy involves harvesting the patient's peripheral blood T lymphocytes, stimulating and expanding the autologous tumour-reactive T cells using IL-2 and CD3-specific antibody, before subsequently transferring them back into the patient. Twelve patients with advanced pancreatic cancer who underwent resection, intraoperative radiotherapy and intraportal infusion of lymphokine-activated killer cells with recombinant IL-2 had lower incidence of liver metastasis compared to controls (3 of 12 vs. 10 of 15; $p < 0.05$) [312]. There was no significant difference in overall survival, but more patients were alive 3 years later (36% vs. none).

Telomerase

Telomerase is a reverse transcriptase that contains an RNA template used to synthesise telomeric repeats onto chromosomal ends. Activation of telomerase and its maintenance of telomeres play a role in immortalisation of human cancer cells, as telomeres shrink after each cell division [313]. Telomerase activity is found in 92–95% of pancreatic cancers [314, 315], and is associated with increased potential of invasion and metastasis and poor prognosis [316, 317]. Upregulation of telomerase may also be responsible for the development of chemotherapy resistance [318]. Adenovirus-mediated transduction of p53 gene inhibited telomerase activity in MIAPaCa-2, SUIT-2 and AsPC-1 cells, independent of its effect on apoptosis, cell growth and cycle arrest [319]. Antisense to the RNA component of telomerase seemed to increase susceptibility of Panc-1 cells to cisplatin [320]. Telomerase reverse transcriptase antisense oligonucleotide (hTERT-ASO) was found to inhibit the proliferation of BxPC-3 cells in vitro by decreasing telomerase activity and increasing apoptosis [321].

Adoptive transfer of telomerase-specific T cells was studied in a syngeneic pancreatic tumour mouse model [322]. T cells were produced in vitro by coculturing human lymphocytes with telomerase peptide-pulsed dendritic cells (DCs) or in vivo by injection of peptide with adjuvant into C57BL/6 mice. Animals treated with these T cells showed significantly delayed disease progression.

MUC1

Adoptive transfer of MUC1-specific cytotoxic T lymphocytes was able to completely eradicate MUC1-expressing tumours in mice [323]. Intraportal infusion of in vitro MUC1-stimulated T cells was performed in patients with pancreatic cancer, with subsequent inhibition of liver metastasis [324]. In a study of 11 patients with lung metastases (from colorectal, pancreatic, breast, lung, or melanoma primaries), effector cells were generated in vitro using cultured DCs, synthetic peptide, peripheral blood lymphocytes, IL-2 and anti-CD3 antibody [325]. A partial response of the lung metastases was observed in a patient with pancreatic cancer who received these cells stimulated with MUC1.

Cytokines and Immunomodulators

TNFerade

TNF-α is a multifunctional cytokine that has shown antitumour potency [326–328]. TNFerade Biologic (TNFerade) is a replication-deficient adenovirus carrying the gene for human TNF-α, regulated by a radiation-inducible promoter early growth response (Egr-1). The latter would ensure maximal gene expression when infected tissue is irradiated [329].

TNFerade was effective in combination with radiation in a number of human xenograft models, including glio-

ma [330], prostate [331], oesophageal [332] and radiation-resistant laryngeal cancers [333]. The multicentre phase II/III Pancreatic Cancer Clinical Trial with TNFerade is currently ongoing and involved patients with locally advanced pancreatic cancer. Patients were given radiotherapy, 5-FU with or without CT-guided transabdominal injection of TNFerade. Preliminary data of 51 patients revealed that the 1-year survival increased from 28 to 70.5% with the addition of TNFerade, with MS of 335 and 515 days, respectively [334].

Virulizin

Virulizin (Lorus Therapeutics Inc.) is a biological response modifier obtained from bovine bile [335]. It stimulates the expression of TNF-α and activates macrophages, which subsequently activates natural killer cells via IL-12 [336, 337]. Evidence exists to show that it also induces the production of IL-17E with resulting eosinophilia [338].

In vivo studies showed that Virulizin significantly inhibited the growth of human pancreatic cancer xenografts (BxPC-3, SU 86.86 and MIAPaCa-2) in nude mice, as well as potentiated the antitumour effect of gemcitabine and 5-FU [339, 340]. A phase III trial was conducted to study the effect of gemcitabine with or without Virulizin in 434 chemotherapy-naïve patients with advanced pancreatic cancer [341]. MS was not significantly better for the gemcitabine and Virulizin group compared to gemcitabine with placebo (6.3 vs. 6 months). However, for stage 3 patients who received Virulizin in a salvage setting, a significant difference in survival was demonstrated (10.9 vs. 7.4 months, $p = 0.017$).

Vaccines

Vaccination involves administering an antigen that is unique for a particular type of tumour with the aim of stimulating tumour-specific immunity. Antigens could be delivered in the form of DNA or peptide, as well as tumour cells or antigen-pulsed DCs. Additional synergistic help is added to elicit a more vigorous and effective immune response, such as cytokines and immunostimulating adjuvants.

Whole Cell

GM-CSF is one of a few cytokines that has shown significant antitumour effect in vivo [342]. It is an important growth factor for granulocytes and monocytes, and has a crucial role in the growth and differentiation of DCs, the most potent antigen-presenting cells (APCs) for triggering immune response. In vivo growth of AsPC-1 cells, retrovirally transduced with the GM-CSF gene, was inhibited and associated with increased survival of the nude mice, even in the mature T cell-deficient condition [343].

Jaffee et al. [342] conducted a phase I study using allogeneic GM-CSF-secreting whole-cell tumour vaccine for pancreatic cancer. This is based on the concept that the localisation of GM-CSF in the implanted tumour environment together with the shared tumour antigen expressed by the primary cancer would effectively induce an antitumour immune response. In this study two pancreatic cancer cell lines (PANC 10.05 and PANC 6.03) were used as the vaccine, both genetically modified to express GM-CSF. Fourteen pancreatic cancer patients who had undergone pancreaticoduodenectomy 8 weeks before were given variable doses of the vaccine intradermally. Three of the 8 patients who received $\geq 10 \times 10^7$ vaccine cells developed post-vaccination delayed-type hypersensitivity (DTH) responses associated with increased disease-free survival time, and remained disease-free for longer than 25 months after diagnosis. Side effects were mainly limited to local skin reactions at the site of vaccination. In a recently completed phase II study of 60 patients with resected pancreatic adenocarcinoma, patients received five treatments of 2.5×10^8 vaccine cells, together with 5-FU and radiotherapy [344]. The reported MS was 26 months, with a 1- and 2-year survival of 88 and 76%, respectively.

Peptide and DNA

Ras

As described earlier, mutated ras is highly prevalent in pancreatic cancer. A phase II study was done using mutant ras peptide-based subcutaneous vaccine in 12 cancer patients (5 with fully resected pancreatic and 7 with colorectal cancers). Five out of 11 patients showed ≥1.5-fold increase in interferon-γ (IFN-γ) mRNA copies in peripheral blood mononuclear cells. The pancreatic cancer patients showed a disease-free survival of >35.2 months and post-vaccination survival of >44.4 months [345].

Gjertsen et al. [346] tested an intradermal vaccine of APCs loaded ex vivo with synthetic ras peptide corresponding to the ras mutation found in the patient's tumour. In this phase I/II study of 5 patients with advanced pancreatic cancer, 2 of them showed induced immune response. They also studied ras peptide in combination with GM-CSF in a phase I/II trial involving 48 patients with pancreatic adenocarcinoma of variable stage [347].

Peptide-specific immunity was induced in 58% of patients. Of patients with advanced disease, those who responded to treatment showed increased survival compared to non-responders (148 and 61 days, respectively; $p = 0.0002$).

As IL-2 is involved in T cell-mediated immune response, a vaccine consisting of mutant ras peptide in combination with GM-CSF and IL-2 was tested in a phase II trial of 17 patients with advanced cancers (14 colorectal, 1 non-small cell lung and 2 pancreatic cancers) [348]. Of the 6 patients with positive immune response (by means of IFN-γ mRNA copies), the MS and the median PFS were 39.9 and 17.9 months compared to 18.5 and 15.6 months for non-responders, respectively. Grade III toxicities led to IL-2 dose reduction in 3 of the patients.

Evidence showed that patients vaccinated with ras peptide developed immunological memory. In a follow-up study of 23 patients vaccinated in 1995–1998, the only 5 survivors were all immune responders during that period (measured as DTH skin reaction and/or in vitro T cell response) [349]. T cell activity, investigated by in vitro T cell proliferation assay, was still present 7–9 years after vaccination.

CEA and MUC1

CEA glycoprotein is expressed at a low level in normal colonic epithelium but is overexpressed in many malignant diseases, including those of the colon, rectum, stomach and pancreas (85–90%) [350]. Its serum level is sometimes used as a marker for the diagnosis of pancreatic cancer, with a sensitivity of 25–40% and a specificity of 70–90% [351–353].

TRICOM is a poxvirus-based vaccine containing tumour-associated antigens in combination with a TRIaid of COstimulatory Molecules (B7-1, intercellular adhesion molecule-1, leucocyte function-associated antigen-3) [354]. The aim is to enhance tumour-specific T cell response. Marshall et al. [355] conducted a phase I study of 58 patients using the replication-defective fowlpox recombinant (rF)-CEA(6D)-TRICOM and recombinant vaccinia-CEA(6D)-TRICOM vaccines, with or without GM-CSF. CEA(6D) contains a modification of the HLA-A2 CEA CAP-1 epitope to enhance its immunogenicity. Only one patient had pancreatic cancer – she was previously diagnosed, had had radiotherapy with chemosensitisation, followed by ALVAC-CEA (replication-defective avipox containing the CEA gene) vaccine because of disease progression [356]. She remained stable for 6 months after ALVAC-CEA but progressed with rising CA 19-9 and pain unresponsive to chemotherapy. After two vaccinations with (rF)-CEA(6D)-TRICOM both CA 19-9 and pain decreased for over a year. Enhanced CEA-specific T cell responses were noted in the majority of patients.

To boost MUC1-specific immune response, a vaccine composed of MUC1 peptide and SB-AS2 adjuvant was tested in a phase I study [357]. There was an increase in the percentage of CD8+ T cells and MUC1-specific antibody (some developed IgG). Hope for the CEA or MUC1 vaccine was nevertheless crushed when a phase III trial of 255 patients using PANVAC-VF (vaccine consisted of recombinant vaccinia and fowlpox viruses coexpressing CEA, MUC-1 and TRICOM) failed to improve overall survival compared to palliative chemotherapy or best supportive care [358].

Gastrin

G17DT (Gastrimmune or Insegia) is an immunoconjugate of the amino-terminal sequence of gastrin-17 (G-17) linked by means of a spacer peptide to diphtheria toxoid. Given intramuscularly it induces the formation of antibodies that can neutralise both amidated-G-17 and the precursor glycine-extended G-17 [359]. In a phase II study of 30 patients, 67% mounted an antibody response [359]. A significantly higher response (82%) was achieved in those given the highest dose of 250 μg compared to 46% in the 100 μg group. MS was significantly higher (217 days) for the antibody responders compared to non-responders (121 days; $p = 0.0023$). When used as a monotherapy for patients with advanced pancreatic cancer unwilling or unsuitable to take chemotherapy, MS was 151 compared to 82 days in the placebo group ($p = 0.03$) [360]. G17DT was subsequently tested in a phase III trial with or without gemcitabine in 383 untreated patients with locally advanced, recurrent or metastatic pancreatic adenocarcinoma [361]. This unfortunately showed that the addition of G17DT did not improve overall survival or secondary endpoints (e.g. response rate, time to progression). Increasing G-17 antibody titre levels in a subset of patients, however, were associated with increased survival.

Mesothelin

Thomas et al. [362] provided the first direct evidence, by using mesothelin epitopes, that pancreatic cancer-specific CD8+ T cell response can be generated via cross-presentation by an approach that recruits APCs to the vaccination site. Gaffney et al. [363] studied the mesothelin DNA vaccine in combination with the anti-glucocorticoid-induced TNF receptor antibody (anti-GITR) in mice with syngeneic mesothelin-expressing pancreatic cancer.

50% of animals treated with mesothelin were tumour-free 25 days after tumour injection compared to 0% of non-treated mice. This increased to 94% with the addition of anti-GITR. The agonist anti-GITR served to enhance T cell-mediated response of the vaccine [364, 365].

Telomerase

The telomerase peptide vaccine GV1001 was tested in a phase I/II study of 48 patients with unresectable pancreatic cancer [366]. They received intradermal injection in combination with GM-CSF. Immune responses, as measured by DTH skin reaction and T cell proliferation in vitro, were demonstrated in 24 of 38 evaluable patients, with the highest percentage (75%) in the intermediate dose group. MS for this group was significantly longer at 8.6 months, and 1-year survival was 25%. GV1001 was given to patients in a phase I trial using imiquimod as an adjuvant [367]. Imiquimod acts by binding to Toll-like receptor 7 on immune cells, resulting in the production of cytokines such as IFN-α, IFN-β and IL-12. Immune response was found in up to 6 (46%) of 13 evaluable patients.

These promising results have led to the commencement of the large, phase III TeloVac trial exploring gemcitabine and capecitabine chemotherapy with concurrent or sequential GV1001 in patients with locally advanced and metastatic pancreatic cancer.

Survivin

Survivin-specific cytotoxic T lymphocytes were isolated from pancreatic cancer patients and these could lyse pancreatic carcinoma cell lines in vitro [368]. Vaccination with survivin DNA prolonged survival in murine pancreatic and lymphoma tumour models associated with slower tumour growth and increased lymphocyte infiltration [369]. Survivin peptide was tested in a patient with gemcitabine refractory pancreatic cancer [370]. Whilst on treatment, he had complete remission of liver metastases after 6 months. However, when he was weaned from the vaccination, he developed recurrent disease. Vaccine-induced immune activity was detected by IFN-γ enzyme-linked immunospot assay.

Antigen-Pulsed DCs

Antigen-specific T cell responses are initiated by DCs. They capture antigens secreted or shed by tumour cells and present peptides in association with the major histocompatibility complex class I and II molecules. This results in the expression and upregulation of cytokines and costimulatory molecules which in turn stimulate CD4+ and CD8+ T cells to mount an antitumour response. As such DCs that carry the tumour antigen of interest are an ideal adjuvant in cancer immunotherapy.

TNP-470

As described previously, TNP-470 is an antiangiogenic inhibitor. Miyazaki et al. [371] tested the combination of DC immunotherapy and TNP-470 in a syngeneic, orthotopic murine pancreatic cancer model. DCs were coincubated with tumour lysates of the murine pancreatic adenocarcinoma cell line Panc02. C57BL/6 mice treated with these DCs showed significant reduction in tumour volume and mean vascular density, associated with prolonged survival. Combination therapy was better than either one alone. Infiltration of CD4+ and CD8+ cells were significantly higher in those treated with tumour lysate-pulsed DCs compared to non-pulsed DCs.

Carcinoembryonic Antigen

In one study, 3 patients with resected pancreatic cancer following neoadjuvant chemoradiotherapy were given monthly injections of autologous, monocyte-derived DCs loaded with the mRNA of CEA for 6 months [372]. No toxicities were reported and all patients remained disease-free for more than 30 months from diagnosis.

MUC1

In a phase I/II trial, human autologous DCs transfected with MUC1 cDNA were used as a vaccine for 10 patients with advanced breast, pancreatic or papillary cancer [373]. Four patients showed a 2- to 10-fold increase in the frequency of mucin-specific IFN-γ-secreting CD8+ T cells, suggesting an immune response. In a phase 1b study, 8 patients with pancreatic or biliary tumours were vaccinated with DCs pulsed with MUC1 [374]. Of the seven serum samples obtained, two showed isotype switching of anti-MUC1 antibodies from IgM to IgG.

Conclusion

Pancreatic cancer is a devastating disease that has a high morbidity and mortality. Early diagnosis and treatment, although difficult to achieve, still offers the best chance of survival. Patients with advanced and metastatic diseases have to rely on palliative treatments that are unpleasant and have limited therapeutic effects. Over the past decades our understanding of this condition has increased tremendously and numerous molecu-

Table 3. Selected pancreatic cancer clinical trials in progress (source: National Cancer Institute)

Phase	Treatment	Disease stage
III	Erlotinib, gemcitabine and capecitabine	Locally advanced/metastatic
	Curcumin, celecoxib and gemcitabine	Locally advanced/metastatic
	Axitinib and gemcitabine	Locally advanced/metastatic
	Sorafenib and gemcitabine	Locally advanced/metastatic
	GV1001 and gemcitabine	Locally advanced/metastatic
	TNFerade, 5-FU and radiotherapy	Locally advanced
II	Curcumin and gemcitabine	Locally advanced/metastatic
	Cetuximab and bevacizumab	Locally advanced/metastatic
	ARQ 197 and gemcitabine	Locally advanced/metastatic
	ARRY-142886 (AZD6244) and capecitabine	(Second line)
	Lapatinib (GW572016) and gemcitabine	Metastatic
	Everolimus (RAD001)	Metastatic
	Sirolimus (rapamycin)	Advanced
I/II	Erlotinib, bevacizumab, gemcitabine and capecitabine	Locally advanced/metastatic
	PTK 787 (ZK222584) and gemcitabine	Advanced
	Survivin peptide	Advanced (also melanoma, colon and cervical cancers)
	Bevacizumab and gemcitabine	Completely removed surgically
	Bevacizumab, 5-FU, oxaliplatin, gemcitabine and radiotherapy	Locally advanced

lar level-targeted biological therapies have been studied. So far not many have made it to the clinical setting, and for those which did, results were far from curative and treatments have to be delivered with standard chemotherapy or radiotherapy for maximum benefits. Nonetheless, multimodality treatments are still the way forward given the complexity but often non-specific nature of tumour compared to normal cells. As such this is still a significant area for extensive research and considerable resources will continue to be channelled into the development of novel biological treatment agents. The results of a number of clinical trials are eagerly awaited (table 3).

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